Cytokine regulation of human leukocyte antigen DRα (HLA-DRα) gene expression in human tumour cell lines

A thesis presented for the degree of PhD in Biological Sciences

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Declaration

All the work reported in this thesis was performed by the author (except where stated) in the laboratory of Dr. Alan Morris in the Department of Biological Sciences, University of Warwick. The described work has not been submitted for a degree at any other institution.

Abbreviations

aa	amino acid
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
bp	base pair(s)
β-gal	β-galactosidase
CAT	chloramphenicol acetyl transferase
CIAP	calf intestine alkaline phosphatase
CRS	*cis*-acting repressor factor
CTP	cytidine triphosphate
DEAE	diethyl amino ethyl
DMEM	Dulbecco's modified Eagle's medium
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediammine tetra acetic acid
EGTA	ethyleneglycol-bis (2'-amino-ethylether) tetra acetic acid
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAS	γ-activated sequence
GBP	guanylate binding protein
GM-CSF	granulocyte monocyte-colony stimulating factor
GTP	guanosine triphosphate
HBS	hepes buffered saline
Hepes	*N*-2-hydroxyethyl-piperazine-*N'*-2-ethansulphonic acid
ICAM-1	intercellular adhesion molecule-1
SV40	simian virus 40
IFN	interferon
IL	interleukin
IPTG	isopropyl-1-thio-β-D-galactopyranoside
IRS	interferon responsive sequence
ISGF-3	interferon stimulated gene factor-3
Jak	Janus kinase
LFA-1	leukocyte function associated antigen-1
LTR	long terminal repeat
MHC	major histocompatibility complex
mRNA	messenger RNA
M_w	molecular weight
NP-40	Nonidet P-40
NRE	negative regulatory element
OD	optical density
ONPG	o-nitrophenyl-β-D-galctopyranoside
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenyl methyl sulphonyl fluoride
poly-(dI:dC)	polydeoxyinosine-deoxycytidine double
(dI:dC)	stranded copolymer
Pol	polymerase
RPMI	Roswell park memorial institute
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
ss	single stranded
STAT	signal transducer and activator of transcription

TBE	Tris-borate electrophoresis buffer
TCR	T-cell receptor
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*, tetramethyl-ethylene diamine
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
tRNA	transfer RNA
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Summary

Class II major histocompatibility complex antigens are cell surface αβ-heterodimeric glycoproteins which function as important immune recognition molecules for the stimulation of $CD4^+$-T lymphocyte-mediated immune responses. Their expression by tumour cells, and the possible implications, has been the subject of much debate. In this investigation aspects of the cytokine-mediated regulation of the transcription of the gene of the human class II MHC molecule HLA-DRα were investigated in human cell lines derived from colorectal and neural tumours.

The transcriptional regulation of HLA-DRα has been shown to be controlled through discrete promoter elements termed the W, X and Y boxes; the W and X elements were investigated here. Nuclear proteins were purified from inducible (colo 201, colo 205 and U373MG) and non-inducible (caco-2 and LS180) cell lines and were shown to associate with oligonucleotide probes corresponding to the W and X regulatory elements of the HLA-DRα promoter using bandshift (gel retardation) experiments. Differences were observed in the populations of W and X box-binding proteins between the cell lines. The IFN-γ treatment of class II inducible colorectal cell lines resulted in the binding of different populations of transcription factors to these probes: novel factors were bound as well as proposed repressor proteins dissociating from these probes.

When a promoter probe corresponding to the 470 base pairs upstream of the cap site was employed, differences in the binding of factors between inducible and non-inducible cell lines were again observed. Treatment of the colorectal tumour cell line colo 205 with IFN-γ resulted in the appearance of a novel binding factor after 6hr treatment. Longer treatment resulted in the net loss of binding factors from the W-X box region in addition to the loss of a postulated "maintenance repressor factor" which associated less specifically with the W-X-Y box region.

The use of reporter gene assays in the glioblastoma cell line U138MG showed that IFN-αβ and TGF-β are both capable of suppression of IFN-γ-induced CAT expression through a 680bp and a 320bp HLA-DRα promoter fragment. The levels of IFN-γ-induced CAT expression were also greater for the 320bp promoter fragment expression vector. The suppression observed was greater with the larger promoter fragment. Concurrent IFN-γ (1U/ml; 48hr) and IFN-αβ (100U/ml; 48hr) reduced DRα680CAT expression to 47% and DRα320CAT expression to 84% of that observed with IFN-γ alone; simultaneous treatment (with 1U/ml IFN-γ and 1000U/ml IFN-αβ) of cells transiently transfected with pDRα320CAT resulted in CAT expression being 26% of that observed with IFN-γ alone. For TGF-β (100U/ml) cotreatment with 1U/ml IFN-γ, the expression of CAT was 27% and 55% of that observed for IFN-γ-treatment alone with pDRα680CAT and pDRα320CAT-transfected U138MG, respectively. It was concluded that the 680bp fragment contained a repressor element absent form the truncated form. The 680bp fragment contained a putative IFN-αβ response element which was removed by its truncation to yield the 320bp promoter region. The colorectal tumour cell lines were unable to express CAT driven by either HLA-DRα promoter fragment. It was hypothesised that a tissue-specific enhancer element was absent from both of these promoter fragments.

This study demonstrated a variety of aspects of the cytokine-mediated regulation of HLA-DRα gene expression. Tissue-specific differences in the regulation of expression were shown between cells of neural and colorectal origin when they were interrogated with regards to their abilities for IFN-γ-induced HLA-DRα promoter-driven gene expression. The binding of nuclear proteins to important regulatory elements of the HLA-DRα promoter also confirmed that tissue- and cell-line-specific differences occurred with this respect.

CHAPTER 1

CHAPTER 1: INTRODUCTION

Opening remarks: The nature and control of gene expression in mammalian cells

Unlike the environment of microorganisms, the internal milieu of higher multicellular organisms such as mammals is, in metabolic terms, very constant. As such, there is little demand for abrupt and substantial changes in the biochemistry of cells of such organisms. There is, therefore, little day to day alteration in the amounts of enzymes of, for example, the glycolytic pathway although short term changes in the activity of the enzymes may occur. However, adaptive changes do occur, and an example of this is the response to the attempted colonisation by pathogenic microorganisms: the immune response. The cells of the immune system can show very large changes in their properties: new functions are induced, and dramatic changes in the physiology of the cells of the immune system do occur. An example is the activation of the resting B lymphocyte to become a metabolically very active plasma cell, secreting large amounts of immunoglobulin.

These changes in cellular physiology are to a large extent due to changes in gene expression. Transcription is a major level at which gene expression is regulated in eukaryotes. It follows that for comprehension of the nature of adaptive changes in the immune system - mammalian cells in general - the elucidation of the mechanisms that control the initiation of transcription is central (La Thangue and Rigby, 1988).

Mammalian gene expression follows the dogma of "transcription" of the gene into message and translation of the message into protein. The structure of mammalian genes is complex, and their organisation into chromosomes adds a further level of complexity: the nuclear location of DNA requires post-transcriptional mechanisms to transport the message to the site of translation. There are elaborate processes to ensure that the resulting protein is delivered to its appropriate location within or without the cell and modified as necessary. Mammalian physiology is highly elaborate, with many different cell systems interacting directly and via diffusible factors to influence each other: and this influence is very much exerted at the level of gene expression to change the constellation of proteins produced within a cell.

It is possible to make a generalisation about the control of gene expression equivalent to the central dogma about the mechanism of gene expression: that is that a primary level of control is through the efficiency of transcription of the gene, this is exerted by the interaction of specific proteins - transcription factors - with particular sequences of the gene - response elements - located usually upstream of the structural gene.

The binding of transcription factors to the promoters of eukaryotic genes during the control of expression is under the control of many stimuli. Certain transcription factors are described as being "general" in that they bind to the same *cis*-acting sequence in many promoters (for example, the TATA and CCAAT boxes). The plasticity of gene expression by eukaryotic cells enables

them to respond to environmental change and as a result they can alter their physiological status to adapt. The maximal control of gene expression often requires the binding of additional transcription factors upstream of the TATA and CCAAT boxes. The binding of additional transcription factors to a promoter sequence is frequently seen to be under the control of stimuli external to the cell.

Described in **section 1.5.** are the mechanisms for the interaction of transcription factors with their specific sequence elements and the subsequent effects on transcription. Many transcription factors only bind to their sequence element upon stimulation by factors exogenous to the cell, for example binding of steroids to their receptor, phosphorylation of CREB after cAMP-induced protein kinase activation. One important type of stimulation is that observed from the binding of cytokines to the specific cell surface receptors of target cells where binding activates cellular genes through intracellular signalling systems targeted on transcription factors (Morris, 1995). The genes induced by cytokines are particularly important in the control of immune responses as well as other non-immunological functions. Hence, the consequences of cytokine-induced gene expression are of great importance to the whole animal.

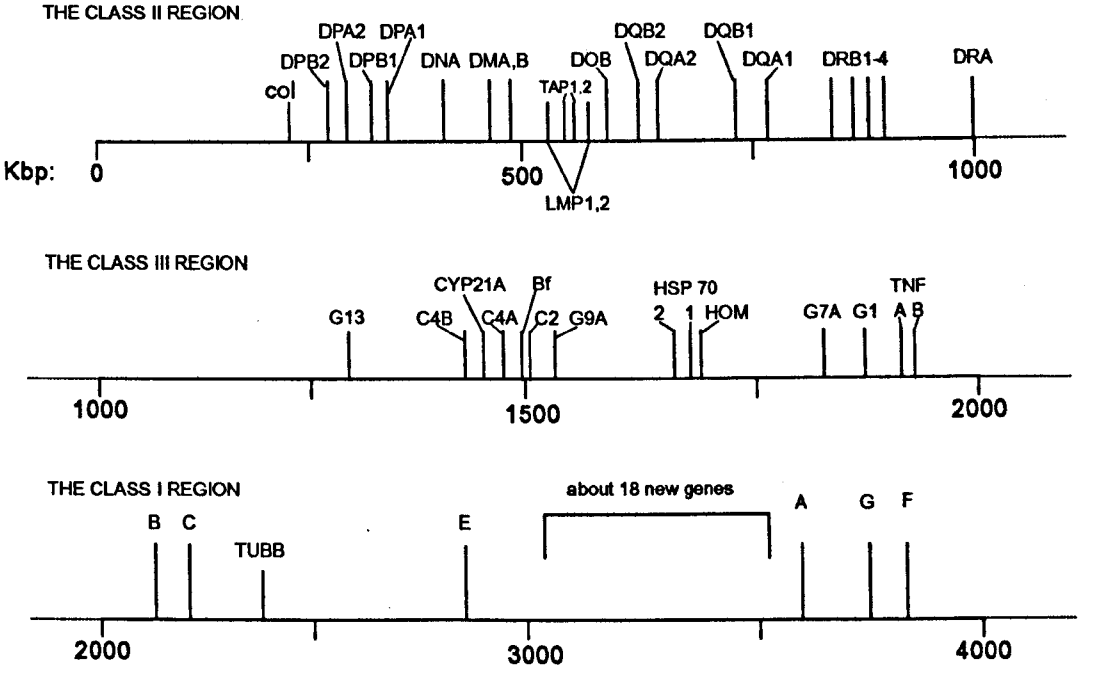
The present study investigated aspects of the rôles of the cytokines, interferon-γ (IFN-γ), interferon-αβ (IFN-αβ) and transforming growth factor-β (TGF-β) in the control of the expression of the gene for the human leukocyte antigen DRα (HLA-DRα). The following sections describe the functions of

immune recognition molecules encoded within the major histocompatibility complex in immune responses. Further on, the transcriptional regulation of genes whose products are essential for an effective immune response against a foreign antigen is discussed.

1.1. Antigens of the major histocompatibility complex of man

Transplantation antigens were initially defined as the cell surface molecules whose polymorphism led to rejection of allogeneic grafts (Gorer, 1937). Such molecules have been found to be encoded in a similar genetic region, called the major histocompatibility complex (MHC), in all vertebrate species examined (Snell, 1981), including man (Dausset, 1981). The MHC is about 4 mega bases in size and encodes about 100 genes. Genes included in this region are those which encode immune response genes (Benacerraf, 1981), which determine an animal's response to a specific antigen (now known to be MHC antigen genes), and some of the components of the complement system. The MHC also encodes genes involved in the processing of foreign antigens, such as those encoding transporter proteins involved in the delivery of peptides to the ER in which their association with MHC antigens occurs (see **figure 1.1.**).

Figure 1.1. Map of the major histocompatibility complex of man



Notes:

The major histocompatibility complex of man is encoded on chromosome 6 and covers approximately 4 megabases of DNA. In this diagram the MHC has been split into class II, III and I regions. Long stalks represent genes for histocompatibility antigens, short stalks are other genes. TAP1 and 2 are genes encoding "transporters associated with antigen presentation", the class II region also encodes proteasome genes. The class III region encodes genes of the complement system: C2, C4A and B and Bf (factor B). This region also encodes tumour necrosis factor α and β (TNF A and B) and genes for heat shock protein 70s. Other genes in the class III region encode genes whose functions are still to be elucidated. The class I region encodes genes for classical class I MHC antigens as well as the β-tubulin gene (TUBB). About 18 new genes have recently been discovered in this region whose functions are yet to be elucidated. Diagram compiled from Morris, *et al.*, 1994.

The best known of the genes of the MHC are those which encode the class I and II histocompatibility antigens. It is these antigens which are responsible for the rejection of allogeneic grafts which first led to their identification. Their physiological rôle, however, is the presentation of peptide antigen to T lymphocytes, which is achieved by the formation of complexes between small peptide fragments of foreign antigen and the histocompatibility antigen expressed at the surface of the antigen presenting cell (APC) where the complex is recognised by the receptor of the appropriate T-cell.

The following sections outline the rôles played by class I and class II MHC antigens in the presentation of peptide antigens to T-lymphocytes and the generation of the subsequent immune responses.

For a T-lymphocyte to be activated, it must recognise antigens presented on the surface of the cell in physical association with the polypeptide products of MHC genes. The rôles of class I and class II MHC molecules is to present antigens in a context recognisable by either of the two major functional types of T-lymphocytes, cytotoxic and helper. Cytotoxic T-lymphocytes (T_c) recognise antigens in association with class I MHC molecules, whereas helper T-lymphocytes (T_h) recognise those antigens in the context of class II MHC.

1.1.1. Class I MHC antigens and $CD8^+$ T-cell responses

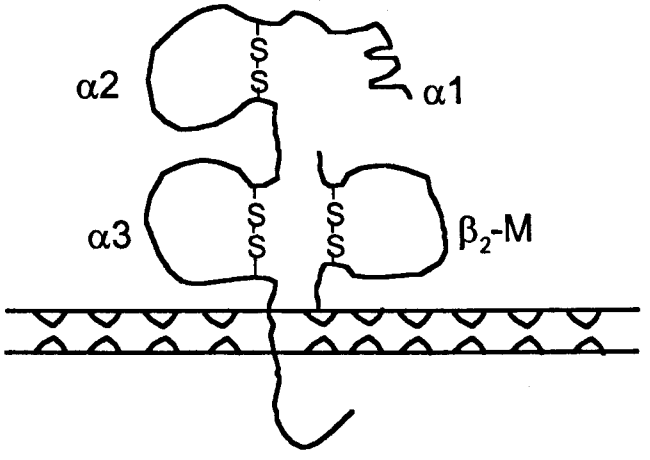
Class I MHC genes encode the α chain of an α/β heterodimer in which the β chain is β_2-microglobulin encoded at a different locus. The classical class I genes (human leukocyte antigens (HLA)-A, -B and -C in man) are involved in the presentation of endogenously synthesised "foreign" antigens to the immune system, for example antigens arising from a viral infection.

The overall structure of class I genes and antigens is strongly conserved between (and within) mammalian species. The proteins are highly polymorphic integral membrane glycoproteins of 44 kD (Ploegh, *et al.*, 1981). The protein structure is immunoglobulin-like and each class I α-chain is defined by five distinct domains, three extracellular "α" domains, a transmembrane sequence and a cytoplasmic region, each of which is encoded by its own exon. It is the diversity in class I MHC structure which allows their association with an equally diverse possibility of foreign peptide antigens.

The formation of the cell surface "class I" molecule requires the α-chain's non-covalent association with the non-polymorphic, non-glycosylated 12 kD β_2-microglobulin polypeptide. This association occurs within the endoplasmic reticulum after peptide binding and is essential for the final folding of the complex and its transport to the cell surface.

A schematic representation of the association of peptide antigen and β_2-microglobulin with a class I molecule is given in **figure 1.2.**

Figure 1.2. Schematic representation of the interaction of moieties in the formation of the class I MHC



Notes:

This diagram represents the major structural domains of class I MHC molecules and the associated β_2-microglobulin (β_2-M). The extracellular domains of the class I MHC molecule are represented by α1-α3. S-S represents the intrapeptide covalent disulphide bonds.

Class I bound peptide epitopes are limited to 8 or 9 amino acids and there appears to be allelic-associated patterns of sequences of naturally bound peptides. Three-dimensional crystal structures of the interaction of class I molecules and peptide antigens have revealed that such association occurs between the class I side chains and the peptide backbone. High resolution data have described six pockets or sub-sites, designated 'A' to 'F', of diverse shape and composition in the antigen-binding groove which might provide specificity for interactions with the peptide side chains (Saper, *et al.*, 1991). Substitution mutations of specific amino acids along the peptide binding groove have allowed the investigation of each of the specificity pockets (Moots, *et al.*, 1991, 1992; Latron, *et al.*, 1991, 1992; Morrison, *et al.*, 1992; Moss, *et al.*, 1991).

Predictions of the properties of Class I-binding peptides are possible from the defined characteristics of these pockets. The elution of natural peptides from Class I MHC molecules and their subsequent sequencing have implicated certain consensus amino acid residues which will allow peptide association in the MHC binding groove. It has been suggested that T-cell receptor (TCR) recognition occurs not only because of specific peptide residues (Falk, *et al.,* 1992; Jardetzky, *et al.,* 1991), but also because of conformational changes induced in the class I molecule by the binding of peptide (Rotzschke & Falk, 1991).

The $CD8^+$ T-cell response

The surface expression of the class I MHC molecule and associated foreign peptide and β_2-microglobulin allows a cell's recognition by $CD8^+$ cytotoxic T cells. Mutational analysis of class I proteins have shown that binding of CD8 with the class I molecule occurs within an acidic loop of the $\alpha 3$ domain (Moots, *et al.*, 1992; Salter, *et al.*, 1990; Connolly, *et al.*, 1990). It has been suggested that the heterodimeric CD8 molecule has the ability to bind two class I MHC at once, enhancing the affinity of the interaction with the TCR. No definitive study has been able to identify the interaction of the TCR with MHC/peptide structure.

Recognition of MHC class I-bearing cells by appropriate $CD8^+$ T lymphocytes is the first step in a chain events which leads to the removal of the antigen-presenting cell. However, the initial contact between T_c and target cell is non-specific, i.e., it does not involve the TCR and target antigen, rather it is mediated by accessory molecules of the T cell CD2 and LFA-1 (leukocyte function associated antigen - 1). The respective target cell ligands for these molecules are LFA-3 and ICAM-1 (intercellular adhesion molecule - 1).

Binding of T_c to the target cell results in its activation through a complex Ca^{2+}-dependent intracellular signalling system transduced from the TCR-CD3 complex. Activation of the T-lymphocyte results in extensive rearrangement of its cytoplasm and the reorientation of the microtubule organising centre and Golgi apparatus towards the contact site between T_c and target cell. Vesicular

components are accumulated in the region in proximity with the contact site. The "lethal hit" is delivered by the secretion of granules containing serine proteases and Ca^{2+} pore-forming proteins assumed to form lethal holes in the membranes of the target cells. The studies leading to these conclusions are reviewed by Terhorst, *et al.*, (1988).

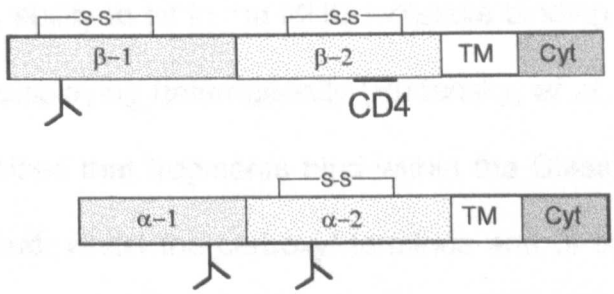
The activation of T-cells leads to production of interleukin-2 (IL-2) which binds to its receptor on the cytotoxic T cell. This binding leads to progression of the lymphocyte through the cell cycle and proliferation (Morris, 1995).

1.1.2. Class II MHC antigens and $CD4^+$ T-cell responses

The class II MHC antigens of man are described by the human leukocyte antigen D (HLA-D) loci (HLA-DR, -DQ & -DP). The proteins are heterodimers consisting of closely related α and β chains whose respective genes are paired within the HLA-D locus. The α and β chains of class II proteins have a domain structure similar to that of the class I MHC α chain in which the polypeptide is split into two extracellular "α" (or "β") regions, a transmembrane sequence and a carboxy-terminal cytoplasmic domain (see **figure 1.3.**). Both chains are glycosylated and are covalently linked intramolecularly via disulphide bonds. The HLA-DR, -DQ and -DP genes display varying degrees of variability, for example, DRβ displays almost all the polymorphism observed for HLA-DR, with 56 different DRβ chains being sequenced (Marsh and Bodmer, 1992), whereas DRα is virtually invariant. However, DQ α and β chains display similar degrees of variance with 12 DQα and 17 DQβ chain sequences (Marsh and Bodmer,

1992; reviewed in Morris, *et al.*, 1994).

Figure 1.3. Domain structures of HLA-D α and β chains



Notes:

A schematic representation of the α and β chains of the class II MHC molecule HLA-DR1. Glycosylation sites are indicated, as are intramolecular disulphide bridges. The probable CD4 binding site within the β-2 domain is indicated. Diagram taken from Morris, *et al.*, (1994).

Class II MHC molecules associate with endocytosed peptide fragments during their processing in the endocytic pathway. The binding of endogenous cellular peptides to the class II MHC is prevented by its association with the invariant chain (Ii) during targetting from the endoplasmic reticulum to the endocytic pathway (Hämmerling and Moreno, 1990).

The peptides bound by class II MHC molecules are generally 17 or 18 amino acids in length (Rudensky, *et al.*, 1991). Such peptides, as a consequence of MHC class II tissue distribution, are derived from exogenous protein antigens which have been internalised by and processed by

macrophages. Interaction between the MHC and peptide appears to be via hydrogen bonding to the backbone of the peptide and the requirement for a specific primary amino acid side chain sequence is under little constraint except for the presence of certain amino terminal "anchor" residues whose presence affects a peptide's ability to sit in the MHC molecule binding groove with the C terminal residues displaying heterogeneity (Rudensky, *et al.*, 1991). Brown, *et al.*, 1993 hypothesised that fragments bind within the Class II groove with the amino terminus fixed, whilst the carboxyl terminus end of the peptide remains open to further trimming by exopeptidases. Amino and carboxy terminal trimming of bound peptides has been shown by other workers (Vignali, *et al.*, 1993; Nelson, *et al.*, 1992), suggesting that both ends of the Class II-bound peptide are open to peptidase action.

The $CD4^+$ T-cell responses

Contacts between $CD4^+$ T cells and antigen presenting cells and B cells are promoted via LFA-1 and CD2 (Figdor, *et al.*, 1990) - as is the case for $CD8^+$ T cell-mediated responses. Interaction between the TCR and MHC class II antigen is optimised by association of CD4.

Recognition of a specific MHC class II antigen complex by the T_h lymphocyte TCR results in signal transduction through the TCR-CD3 complex leading to T cell activation as well as activation of the APC. Activation of macrophages results in production of IL-1. Interleukin-1 stimulation of T_h results in their ability to respond to IL-2 stimulation such that they themselves express

IL-2 and its receptor. The secretion of IL-2 by activated T-cells is wide-reaching and will promote the proliferation of any T-cell which has receptors for IL-2.

Hence, activation of T_h via their interaction with class II MHC presenting specific peptide antigen results in the stimulation and recruitment of many T-cells such that the antigen can be eliminated.

The above accounts of T cell activation are simplified (Roitt, 1994), yet they illustrate the necessity of the recognition of antigens in the context of the MHC in order that a complete immune response may be mounted. The following sections describe the tissue distribution and regulation of expression of MHC antigens and the regulation of their expression both at the cellular and genetic level.

1.2. Tissue distribution of MHC molecules

1.2.1. Constitutive expression of MHC antigens

Constitutive expression is defined here as the surface expression of antigens by cells which is observed in the absence of cytokines, or other induction brought about by viral infection or any other external stimuli. Such knowledge was gained by the staining of tissue sections with monoclonal antibodies against framework determinants of class I or II MHC antigens. Due to the inducibility of MHC antigens, the possibility of false positives due to local inflammation in tissues should not be ignored.

In humans the class I MHC antigens, HLA-A, -B, -C, are expressed by a wide range of cell types including nearly all types of haematopoietic cells (except erythrocytes), fibroblasts, endothelial cells, keratinocytes, peripheral neurones and most epithelial cells. Few tissues contain cells which do not express class I, these include the central nervous system neurones, hepatocytes and some glandular epithelial cells.

Antigens encoded by the HLA-D loci are expressed by a restricted group of tissues. Constitutive expression of class II MHC molecules is only observed by B cells and dendritic cells.

1.2.2. "Induced" expression of MHC antigens

Although normal tissues have a restricted expression of MHC antigens, especially in the case of class II, their expression can be augmented or upregulated in various situations.

1.2.2.1. Infection

The most common of these is infection where MHC antigens are produced in response to foreign antigens. Here, local production of cytokines, in particular interferons (IFNs) -αβ and -γ, results in augmentation of class I (IFN-αβ and IFN-γ) and class II MHC (IFN-γ, alone). The induction of class II MHC antigens by IFN-γ occurs in the cells of many tissues, for example, activated T-cells, macrophages, granulocytes, endothelial cells, keratinocytes, fibroblasts, glandular epithelial cells and glial cells. In the case of infection, the expression of MHC antigens by cells is critical in the removal of the pathogen as discussed in **section 1.1.** above. Here, class I expression by virally infected cells results in their removal by cytotoxic T cell action. The interaction of soluble antigen with specific membrane-bound immunoglobulin of B cells results in its internalisation and processing into the class II MHC-associated surface antigen resulting in the $CD4^+$ T_h response.

1.2.2.2. Autoimmunity

Certain autoimmune conditions have been associated with the inappropriate expression of MHC antigens (Feldmann, 1989). Here it is assumed that MHC molecules become associated with "self" antigens and their

expression at the cell surface invokes the local immune response against the cell. HLA expression or overexpression has been implicated in ulcerative colitis (McDonald and Jewell, 1987), autoimmune thyroiditis (Piccinini, *et al.*, 1987), and multiple sclerosis (Traugott, 1987). In all of these cases, class II (and class I in multiple sclerosis glial cells) are expressed where the cells involved are usually negative. This expression of MHC antigens is also associated with heavy infiltration of the affected tissues by inflammatory cells (reviewed in Morris, *et al.*, 1994).

1.2.2.3. Malignant disease

In malignant disease class I antigens may be lost by tumour cells such that non-expressing cells within a tumour will be "selected" for due to their evasion of detection by cytotoxic T cells. Tumour cells may also gain class II expression. This is usually restricted to a few cells within the tumour in usually less than half of the incidences of a particular cancer.

In the case of colorectal carcinoma, studies have shown the induction of HLA-DR in greater than half of the tumours investigated (Möller, *et al.*, 1991), whereas HLA-A, -B, -C expression was absent in 9% and reduced in 35% of tumours. This observation, supported by other studies, is indicative of the expression of MHC antigens being affected by the neoplastic state and/or the infiltration of tumours by lymphocytes; the colon epithelium is usually negative for class II MHC, yet is constitutive for class I expression. Donnellan, *et al.*, (1995) showed that the ability of colorectal tumour cells to express HLA-DR is

abolished despite the infiltration of the tumour by activated T cells.

The implications of MHC antigen expression by tumour cells for prognosis are unclear and conflicting evidence exists as to whether MHC expression leads to a favourable outcome. For example, in melanoma HLA-DR expression by primary tumours was associated with poorer prospects of disease-free survival than those primaries which were negative for HLA-DR (Zaloudik, *et al.,* 1988). In contrast, loss of MHC class II antigens by B cell lymphomas correlates with significantly shorter survival (Spier, *et al.,* 1988). In the case of colorectal carcinoma, expression of MHC antigens appears to be of little consequence in the prognosis of patient outcome.

1.3. Regulation of MHC class II antigen surface expression

The above section described how the surface expression of MHC antigens is modulated in certain conditions of infection or disease. Of interest is the regulated expression of class II MHC antigens since they are only observed in a limited selection of tissues prior to their "induction" and their augmented expression can have the implications mentioned in **section 1.2.2.** Expression of class II MHC antigens is also modulated on haematopoietic cells as well as those from non-haematopoietic origin, this modulation is discussed in the following sections.

1.3.1. Cytokine mediated class II MHC expression

Cytokines can be considered as polypeptide "hormones" which have a short-range action in, generally, mediating immune responses. They are produced largely by cells of the immune system such as activated T lymphocytes and macrophages (Morris, 1995). Due to the augmented expression of MHC antigens at the sites of inflammation, and the presence of activated T cells and macrophages therein, much research into regulation of MHC expression has concentrated on cytokines although these are by no means the only physiological factors which have a rôle in this regulation.

The most potent inducer of class II MHC antigen expression is interferon-γ (IFN-γ) which can affect many cell types of haematopoietic and non-haematopoietic origin (reviewed in Morris, *et al.,* 1994 and Cogswell, *et al.,*1991). Class I MHC antigens can be induced on many cell types by IFN-αβ as well as IFN-γ.

Other cytokines having a positive effect on MHC class II expression are: interleukin-4 (IL-4) in B cells; granulocyte-monocyte colony stimulating factor (GM-CSF) in monocytes; tumour necrosis factor-α (TNF-α) can synergise with IFN-γ to increase induced class II in a variety of cell types.

A number of cytokines have been identified which have a negative effect on the expression of class II MHC. Although IFN-αβ induces class I expression, it is a potent inhibitor of class II expression (Morris and Tomkins, 1989).

Transforming growth factor-β (TGF-β) is an inhibitory factor produced by many cell types which displays immunosuppressive (Wrann, *et al.,* 1987) as well as growth-*inhibitory* effects on many untransformed cell types (Morris, (1995)). This "cytokine" has been shown to inhibit the IFN-γ-induced surface expression of HLA-D in many human tumour cell lines (Darley, *et al.,* 1993). TGF-β has been shown to be produced by many cell types including activated T lymphocytes (Morris, (1995)) and certain tumour cells (Barrett-Lee, 1990; Coffey, *et al.,* 1987) and its expression has been demonstrated to be altered by activated *ras* oncogene (Glick, *et al.,* 1991). The implications for such a potent immunosuppressor and growth inhibitor to be produced within tumours are vast in that any immune surveillance by tumour infiltrating lymphocytes might not be possible due to the TGF-β-inhibited expression of HLA-D by the tumour cells. This digression illustrates the interaction between tissues, and the cytokines ("growth" factors) which they produce, in the regulation of MHC class II expression.

1.3.2. The effects of non-cytokine factors on class II MHC expression

The effects of other physiological molecules upon the IFN-γ-induced expression of class II MHC antigens has been studied. Hormones of the endocrine system; noradrenaline (Loughlin, *et al.,* 1993), thymosin (Baxevanis, *et al.,* 1992), and thyroid stimulating hormone (Todd, *et al.,* 1987), have all been shown to augment IFN-γ-induced expression. Conversely, corticosteroids suppress macrophage expression, and induced MHC expression has been

shown to be inhibited by growth factors (Todd, *et al.*, 1990), prostaglandins (Snyder, *et al.*, 1982; Ivashkiv and Glimcher, 1991) and lipopolysaccharide (Koerner, *et al.*, 1987).

From the evidence cited above, it is clear that modulated expression of class II MHC antigens in human tissues is a complicated situation orchestrated by many participants which interact synergistically or in an inhibitory fashion with the major inducer IFN-γ. Not mentioned, but implicated, is the altered expression of HLA by cells in the neoplastic state. The presence of the activated *ras* oncogene has been associated with up-regulation of class II MHC in melanocytes (Albino, *et al.*, 1986) class II negative variants of B-lymphoblastoid lines (Kara and Glimcher, 1991; Hume, *et al.*, 1987) and murine primary fibroblasts (Darley and Morris, 1993). Activated *ras* is present in many tumours ranging from 90% of adenocarcinomas of the pancreas to 30% of lung tumours and myeloid leukaemias (Forrester, *et al.*, 1987; Bose, *et al.*, 1987; Bos, 1989;). Transfection of the *c-Ha-ras* oncogene into a poorly differentiated colorectal carcinoma cell line rendered it resistant to natural killer (NK) cell lysis (Bagli, *et al.*, 1990). The influence of MHC antigen expression by colorectal tumours on survival was reported by Möller, *et al.*, (1991) where it was concluded that loss of expression of these molecules bared little relevance to patient survival. Therefore, the regulation of class II MHC antigen expression by non-haematopoietic cell types is of interest due to the implications for diseases apart from their rôle in the presentation of foreign antigens to T-lymphocytes in the eradication of a pathogen.

1.4. Mechanisms for the cellular action of interferons (IFNs)

The following sections will go on to discuss the mechanisms by which the expression of the human class II MHC gene HLA-DRα is regulated at the genetic level. Of interest is the IFN-γ-induced expression, and to appreciate the complicated nature of this gene's activation by IFN-γ, it is necessary to describe the current knowledge of the IFN-γ-induced transcription of genes.

1.4.1. Signal transduction through the interferon receptors

A direct signal transduction mechanism pathway leading from the receptors for IFN-α and IFN-γ has been recently elucidated (Darnell, *et al.*, 1994). Binding of IFN-γ or IFN-α to its respective receptor results in the phosphorylation of a population of proteins at the cytoplasmic face of the transmembrane receptor.

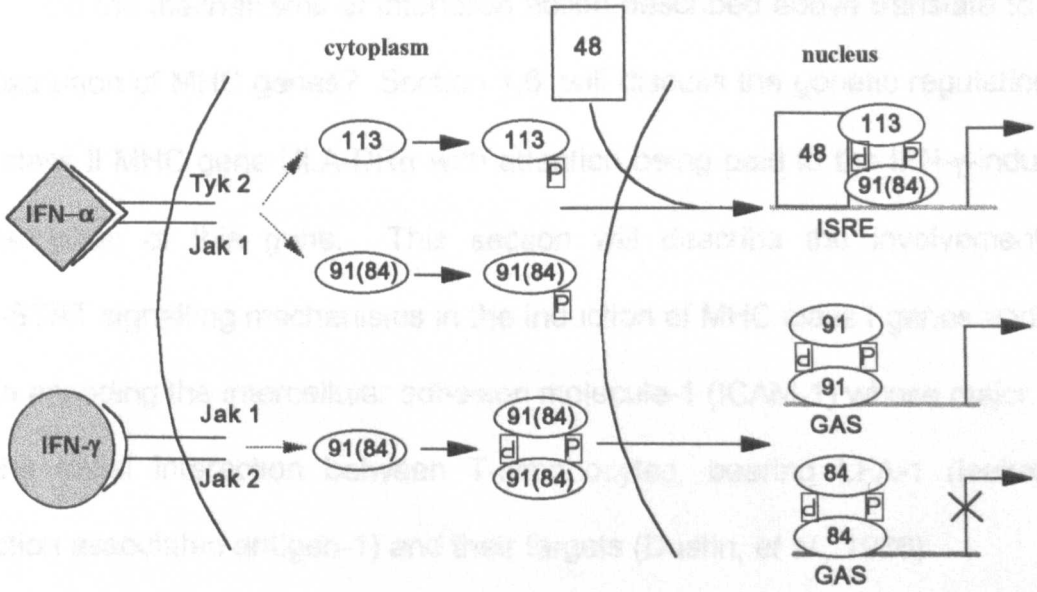
IFN-α stimulation results in the activation of Jak 1 (Janus kinase) and Tyk 2 by tyrosine phosphorylation (Müller, *et al.*, 1993). These activated proteins then mediate tyrosine phosphorylation of 113kD, 91kD and 84kD proteins (named p113, p91 and p84 respectively) of the α-subunit of interferon stimulated gene factor-3 (ISGF-3; Fu, *et al.*, 1990). The phosphorylated moieties p113 and p91 (or p84) dimerise and combine with the γ-subunit, termed p48, and the trimeric protein translocates across the nuclear membrane where it can activated gene transcription through interferon response elements

in the promoters of responsive genes (Müller, *et al.*,1993; Darnell, *et al.*, 1994).

The stimulation of cells with IFN-γ involves similar proteins from the same "family". Interferon-α and -γ stimulation of the guanylate binding protein gene was investigated by Decker, *et al.*, (1989). Subsequently IFN-γ-mediated activation of a cytoplasmic DNA-binding factor (termed GAF for "gamma activated factor") was implicated as a primary step in the stimulated expression of this gene (Decker, *et al.*, 1991). The binding of IFN-γ to its receptor results in the phosphorylation of p91, or its truncated splice-variant p84, which is mediated by activated Jak 1 and Jak 2 tyrosine kinases. Phosphorylation results in dimerisation and translocation into the cell's nucleus where it binds to γ-activated sequences (GASs) in the promoters of responsive genes (Darnell, *et al*, (1994)).

The mechanism by which Jak-family tyrosine kinase-mediated signal transduction results in the transcriptional response within the nucleus has led the mediators, e.g. ISGF-3 and GAF, to be termed generically as "STATs" for signal transducers and activators of transcription. A schematic diagram representing the interplay between receptor binding, phosphorylation and gene activation is given in **figure 1.4.**

Figure 1.4. Representation of the Jak-STAT signal transduction pathway for the interferons



Notes:

The binding of IFN-α and IFN-γ to their respective cell surface receptors activates the protein tyrosine kinases Jak 1, Tyk 2 (for IFN-α) and Jak 1 and Jak 2 (for IFN-γ) by phosphorylation. Tyrosine phosphorylation (indicated by "P") of STAT proteins p91 (p84), and p113 leads to their respective interactions with each other, and p48 (for IFN-α) prior to their translocation into the nucleus to activate transcription of the appropriate genes.

ISRE=interferon stimulated response element; GAS=gamma activated sequence

Diagram reproduced from Darnell, *et al.*, (1994)

From the work by researchers such as Darnell, many questions have been answered with regard to the mode of action of the interferons. A number of members of the STAT family of proteins have been elucidated and other peptide ligands also use this mechanism to exact the appropriate cellular response.

1.4.2. Jak-STAT signal transduction pathways and interferon-induced expression of class I MHC and ICAM-1

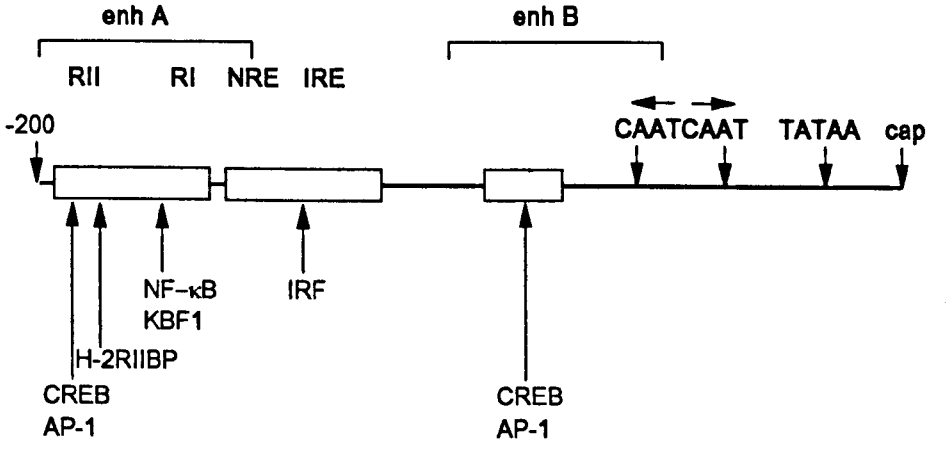
Do the mechanisms of interferon action described above translate to the transcription of MHC genes? Section 1.6. will discuss the genetic regulation of the class II MHC gene HLA-DRα with attention being paid to the IFN-γ-induced transcription of this gene. This section will describe the involvement of Jak-STAT signalling mechanisms in the induction of MHC class I genes and the gene encoding the intercellular adhesion molecule-1 (ICAM-1) whose major rôle is the initial interaction between T-lymphocytes, bearing LFA-1 (leukocyte function associated antigen-1) and their targets (Dustin, *et al.*, 1986).

Class I MHC

The genetic structure of class I MHC promoters is well defined and a number of protein transcription factors which bind to discrete sequence elements have also been elucidated. Class I gene expression can be induced by either IFN-αβ or IFN-γ and the promoter contains a consensus interferon response element which has been shown to bind IRF (interferon regulatory factor). This factor is defined as one of the p48 family of transcription factors in Darnell, *et al.*, (1994). IFN-γ also has a rôle to play in the activation of class I MHC genes, yet there is no consensus element for the binding of p91 or any other defined IFN-γ-induced factor to this promoter. It is possible that IFN-γ treatment of cells results in the activation of the gene encoding a secondary transcription factor(s) which then mediate transcription of class I, such a possibility is borne-out by the requirement for *de novo* transcription. Figure 1.5

is a schematic representation of class I MHC promoters indicating some of the transcription factors which are known to be involved in transcriptional regulation.

Figure 1.5. Schematic representation of class I MHC promoters



Notes:

Important regions of nuclear factor binding sites are indicated by blocks. Enh A B are enhancers A and B. RI and II and regions I and II of enhancer A; NRE is a negative regulatory element; IRE is the interferon response element. The transcription factors and their approximate regions of association are indicated beneath the promoter structure.

Redrawn from Morris, *et al.*, (1994)

ICAM-1

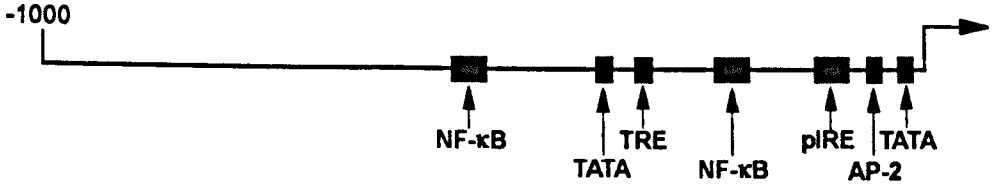
The intercellular adhesion molecule-1 (ICAM-1), like class II MHC antigens, is expressed on limited number of cell types but its expression can be induced by a number of inflammatory cytokines including IFN-γ, TNF-α and IL-6. The regulation of gene expression with respect to cytokine-induced expression has been fruitful in determining the mechanisms by which induced expression is brought about at the genetic level.

The promoter for the human ICAM-1 was cloned and sequenced as reported by Stade, *et al.,* (1990). The region of DNA cloned spanned the nucleotides from -477 to +700, relative to the proposed cap site, and putative consensus sequences for the binding of transcription factors were identified. The proposed sequences for the binding of transcription factors included those for SP-1, AP-1, NF-κB as well as interferon and retinoic acid response elements, these sites being dispersed through the 5' untranscribed promoter region and with proposed NF-κB, SP-1 and retinoic acid receptor binding sites occurring within the first intron.

Since this initial report, much research has concentrated upon determining the functional promoter elements involved in this gene's regulation. An IFN-γ-responsive region was identified which also serves as an IL-6 response element. This palindromic sequence, termed pIRE, shows close homology to the defined γ-activated sequence (GAS) of the human guanylate binding protein gene. It has been determined that the 91kD component of

ISGF3 is induced by both IFN-γ and IL-6 in the induction of ICAM-1 expression (Caldenhoven, *et al.*, 1994; Look, *et al.*, 1994). A schematic diagram of the ICAM-1 regulatory sequence is shown in **figure 1.6.**

Figure 1.6. Schematic representation of the ICAM-1 promoter region



Notes:

The potential *cis*-acting elements of the ICAM-1 promoter, indicated by solid blocks, are: TATA boxes, TPA-responsive element, AP-2, NF-κB, and pIRE site. The start of transcription is indicated by the arrow.

1.4.3. Summary

The interferon-induced gene expression of MHC class I antigens and ICAM-1 involves the binding of STATs to defined regulatory elements of promoters. These factors are members of a growing family which transduce the IFN signal from its receptor at the plasma membrane to the nucleus to control gene expression. Activation of STATs requires their phosphorylation by Jak tyrosine kinases, themselves activated by phosphorylation upon binding of ligand to its receptor. The following sections will describe how there is no clear involvement of Jak-STAT signalling pathways in the IFN-γ-induced expression of class II MHC genes, specifically that encoding HLA-DRα.

1.5. Regulation of gene expression at the transcriptional level

Before describing the current knowledge of the transcriptional regulation of HLA-DRα gene expression, this section will describe the types of transcription factors known to associate with regulatory sequences of eukaryotic promoters and how this binding occurs.

1.5.1. Transcription factor interactions with DNA

Major motifs which are essential for the binding of transcription factors with *cis*-acting response elements have been identified after nuclear factors have been purified, cloned and sequenced. Other sequence motifs of transcription factors have been identified which are responsible for their association with other transcription factors and "transactivation". The following sections summarise some important elements of protein-DNA and protein-protein interactions which influence the events at the start of transcription.

DNA binding motifs

i) Helix-loop-helix

This motif was first identified in bacterial DNA regulatory proteins such as Cro and were characterised by X-ray crystallography. Studies into the binding of eukaryotic transcription factors have identified this motif in a number of transcription factors. In eukaryotes, this binding motif was first identified in *Drosophila* homeotic genes which specify master regulatory proteins controlling

the expression of body plan genes. As a result, the DNA and amino acid sequence of a transcription factor which contains a helix-loop-helix motif is called the *homeobox.* This region contains many basic amino acids which enables interaction with the DNA phosphate moiety. It appears that one of the α-helices recognises the nucleotide sequence of the DNA while the other interacts with the phosphate-sugar backbone (see Struhl, 1989; Dressler, 1989; Marx, 1988). The helix-loop-helix DNA binding motif has been identified in a number of mammalian transcription factors including members of the POU (Pit 1, Oct 1/2, Unc 80) family (for review see Faisst and Meyer, 1992).

ii) Zinc finger proteins

This type of DNA binding motif was discovered in TFIIIA of *Xenopus* whose activity is dependent on and which contains zinc (Struhl, 1989; Evans and Hollenberg, 1988). In this type of DNA binding motif zinc-stabilised loops are formed by the coordination of the metallic ligand between cysteine and histidine residues within the amino acid sequence of the polypeptide. This type of transcription factor has a variable number of zinc fingers which interact with DNA in a manner such that they interdigitate with the sequence element, with adjacent fingers making contact with opposite sides of the DNA helix. Many zinc finger proteins have been identified, not all of which are involved in DNA binding, including SP1, steroid, thyroid and retinoid receptors in mammals and the adenovirus-encoded E1A protein.

iii) Leucine zippers

Certain transcription factors contain a domain which is required for their interaction with other, similar, proteins. This domain has been described as a leucine zipper which consists of a 35aa α-helix with every seventh residue being a leucine (Struhl, 1989; Landschultz, *et al.*, 1988). This can interact with a similar helix form a another protein molecule to "zip" together and form a dimer. The DNA-binding domain is adjacent to the α-helix and can only associate with the sequence element upon dimerisation. Dimers can be hetero- or homodimeric, for example the fos/jun heterodimer or the jun/jun homodimer forming the transcription factor AP1.

1.5.2. Activation of transcription

Thus the sequence-specific interactions of transcription factors with response elements can be mediated by an array of DNA-binding motifs conferred upon the complex structures of proteins. However, the binding of transcription factors alone does not result in transcriptional effects, transcription factors must also interact with each other through their "transactivation" domains to exert their effects. Transactivating domains can be amphipathic helices which were first characterised for the yeast factors GCN4 and GAL4 (La Thange and Rigby 1988), whereas glutamine-rich regions have been described for SP1, Oct1/2 and Pit 1 and proline-rich regions are known for AP1 and the CTF family of transcription factors. It is thought that transcriptional activation is mediated by the protein-protein or protein-RNA polymerase interactions through their transactivation domains.

The mechanism of the action of the transcription factor ATF has been described (Horikoshi, *et al.,* 1988; and Hai, 1988) and it was shown that ATF association with its consensus element allows weakly bound TFIID to associate strongly at the TATA box and the transcriptional start site. Once the increased TFIID association occurs, the binding of RNA polymerase and other TFIIs is facilitated and transcription can occur from this stable complex.

1.6. Regulation of HLA-DRα gene expression at the molecular genetic level

1.6.1. HLA-DRα expression is under transcriptional control

The regulation of HLA-DRα expression is controlled at the transcriptional level (Cogswell, *et al.,* 1991; Sullivan, *et al.,* 1987; and Benoist and Mathis, 1990). The induction of HLA-DRα expression by IFN-γ results in the accumulation of cytoplasmic mRNA transcripts after 4 to 8hr which reaches its maximal level after 24hr (Collins, *et al.,* 1984; and Rosa, *et al.,* 1983). The cell surface expression of HLA-DR reaches maximal levels by 48hr (Rosa, *et al.,* 1983).

Transcription (the synthesis of an mRNA molecule complementary to the sequence of DNA) of eukaryotic genes is mediated by the binding of specific DNA-binding proteins, called transcription factors, to sequences of DNA in the region of genes upstream of the transcriptional start site - the promoter. The transcription of most genes is mediated through the TATA box, necessary for

the accurate positioning of the initiation site transcription, and transcriptional initiation occurs after the association of TFsIIA, D, E and B and RNA polymerase II, (La Thangue and Rigby, 1988). As described in **section 1.5.,** the control of transcription is mediated by the binding of promoter-specific transcription factors to *cis*-acting sequence elements upstream of the TATA box. These transcription factors act in concert to affect the events at the TATA box. Transcription factors may also be repressor proteins which function to regulate transcription in a negative manner.

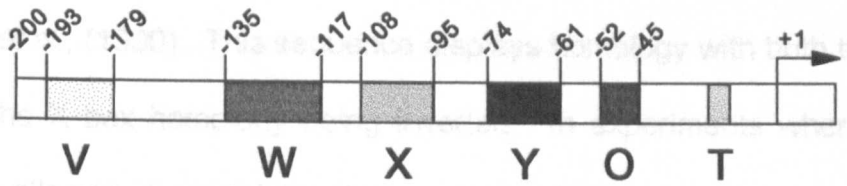
1.6.2. Structure of the HLA-DRα promoter

Early studies into the transcriptional regulation of HLA-DRα (and other human and rodent class II MHC) gene expression relied on sequence data of the region of DNA 5' to the structural gene. The available sequences for all class II genes were compared to determine whether there were homologies between them. It was found that the class II genes contained conserved DNA sequence elements in their promoter regions and some of which show homology to *cis*-acting sequences of other genes, including a TATA box (TATTA) (Sullivan *et al.,* 1987). The original "class II box" was defined by two highly conserved 13 base pair elements, termed X and Y, which are separated by 19-20bp of DNA (Okada, *et al.,* 1985). In HLA-DRα, the X box (CCTAGCAACAGATG) is found between nucleotide positions -108 and -95 and the Y (CTGATTGGCCAAAG) box occurs between nucleotides -74 and -61. The Y box contains an inverted CCAAT box (ATTGG), a transcriptional activator to which ubiquitous nuclear factors generally bind. Peculiar to the HLA-DRα

promoter is an octamer motif (ATTTGCAT) at position -52 to -45. A third class II sequence element was defined upstream of the X box, originally termed the Z box (H or S in murine genes), the W box (TCCTGGACCCCTTTGCAAGA) is comprised of 18 nucleotides from position -135 to -117 in the promoter of HLA-DRα. This element contains two so-called Servenius sequences of which SRV1 shows an inverted homology to the X box (Cogswell, *et al.*, 1991a). The V box (GTTGTCCTGTTT) covers the nucleotides form -193 to -179. **Figure 1.7.** represents the structure of the HLA-DRα promoter.

The promoter of the class II invariant chain has also been shown to contain sequence elements homologous to those found in class II MHC promoters (Brown, *et al.*,1991). This appears to be a consequence of the requirement for its coordinate regulation along with class II α and β chain genes.

Figure 1.7. The structure of the HLA-DRα promoter



Notes:

The proposed regulatory elements of the HLA-DRα promoter defined by sequence homology with other class II MHC genes. V, W, X and Y are all MHC class II-specific sequence elements. "O" represents an octamer motif and T is the TATA element. The nucleotide ranges of each element is given respective to the cap site (start of transcription, designated as +1).

1.6.3. Functions of *cis*-acting sequences

By deletional and sequence mutagenesis of the HLA-DRα promoter and its employment in reporter gene assays, the functions of the *cis*-acting elements, V, W, X and Y, were assessed in cell lines. Such assays rely on the expression of a gene (introduced into the cell by transfection in plasmid form) which is usually absent from the cells studied. In many cases the bacterial gene for chloramphenicol acetyl transferase (CAT) is employed and its expression is driven by the heterologous promoter being studied. Reporter gene assays, therefore, allow the manipulation of a promoter such that the functions of individual regions can be assessed in the activation of gene expression. The following sections will describe the individual functions of the defined *cis*-acting elements and then will discuss how they work in concert to bring about tissue-specific and IFN-γ-induced expression of HLA-DRα.

i) The V box.

The V box was delineated as a weak negative regulatory element by Cogswell, *et al.,* (1990). This sequence displays homology with both the W and X boxes, the X box homology being inverted. In experiments where nuclear factors are allowed to associate with a radiolabelled probe corresponding to a particular DNA sequence, called bandshift, gel retardation or electromobility shift assays, the V box has been shown to form two protein-DNA complexes. The proteins which bind to the V box have not been identified.

ii) The W box

The W box has been shown to be essential for tissue-specific and IFN-γ-induced transcription of HLA-DRα (Tsang, *et al.,* 1988; Cogswell, 1991b). Its involvement in the IFN-γ-induced expression was shown to be critical by Basta, *et al.,* (1988): when the region of DRα from -141 to -109 (which contains the W box) was mutated, IFN-γ-inducibility of reporter gene expression was abolished.

In bandshift experiments the W box had been shown to bind two nuclear factor, termed W-B1 and W-B2 (called NF-Zc and NF-Z2 by a different group) (Tsang, *et al.,* 1990; and Cogswell, *et al.,* 1990a and 1990b). These factors were also shown to be competed out by an X box oligonucleotide - a consequence of their binding to the X box homology within the W box.

iii) The X/X_2 box

This element is also essential for tissue-specific and IFN-γ-induced HLA-DRα expression (Tsang, *et al.,* 1990; Sherman, *et al.,* 1987; and Sherman, *et al.,* 1989).

The X box can be dissected into two elements, termed X and X_2, the latter being defined after it was shown to bind a leucine zipper type of transcriptional activator, termed human X box binding protein 1 (hXBP1) (Liou, *et al.,* 1990) later shown to form a heterodimer with *c-fos* (Ono, *et al.,* 1991a & b). The X_2 box has homology with the AP-1 binding site and, not surprisingly, was shown to bind the *jun-fos* heterodimer (Peterlin and Andersson, 1990).

The X box has been shown to bind a factor termed regulatory factor-X (RF-X) which has been shown to be absent in the condition bare lymphocyte syndrome (Reith, *et al.,* 1988). However, this factor's involvement in this disease in now in dispute (reviewed in Ting and Baldwin, 1993). An IFN-γ "enhanced" factor from murine primary astrocytes has been shown to associate with the HLA-DRα X box (Moses, *et al,* 1992), this factor has been termed IFNEX for "IFN enhanced X" box factor.

iv) The Y box

This sequence element has also been described as being critical for tissue-specific and IFN-γ-induced HLA-DRα gene expression (Tsang, *et al.,* (1990); Sherman *et al.,* (1989)). Its spacing from the X box is conserved at 18

to 20 nucleotides, but the spacer's sequence is variable (Kelly and Trowsdale, 1985). The critical spacing was shown to enable the alignment of X and Y box binding factors to affect transcription (Vilen, *et al.*, 1991).

The Y element contains an inverted CCAAT box which interacts with several DNA binding proteins, the first of these whose gene was isolated was termed YB-1 (Didier, *et al.*,1988). This factor binds to the CCAAT element of HLA-DRα and is thought to be a negative factor since levels of its mRNA are inversely correlated with class II expression in several cell lines (Didier, *et al.*, 1988).

The DRα Y element binding protein (YEBP), also called NF-Y, has been established as a CCAAT-element-binding protein by methylation interference analysis (Sherman, *et al.*, 1989). The relationship between NF-Y association with the Y box and the upstream X/X_2 element was demonstrated by Wright, *et al.*, (1994). By *in vivo* genomic footprinting, it was shown that mutation of the CCAAT box abolished binding at the X/X_2 element, whereas mutation of the X/X_2 box had no effect on CCAAT box binding. Thus binding of NF-Y at the CCAAT box stabilises association of factors at the X box and as such is an initial event in the association of transcription factors with the HLA-DRα promoter.

v) The octamer element

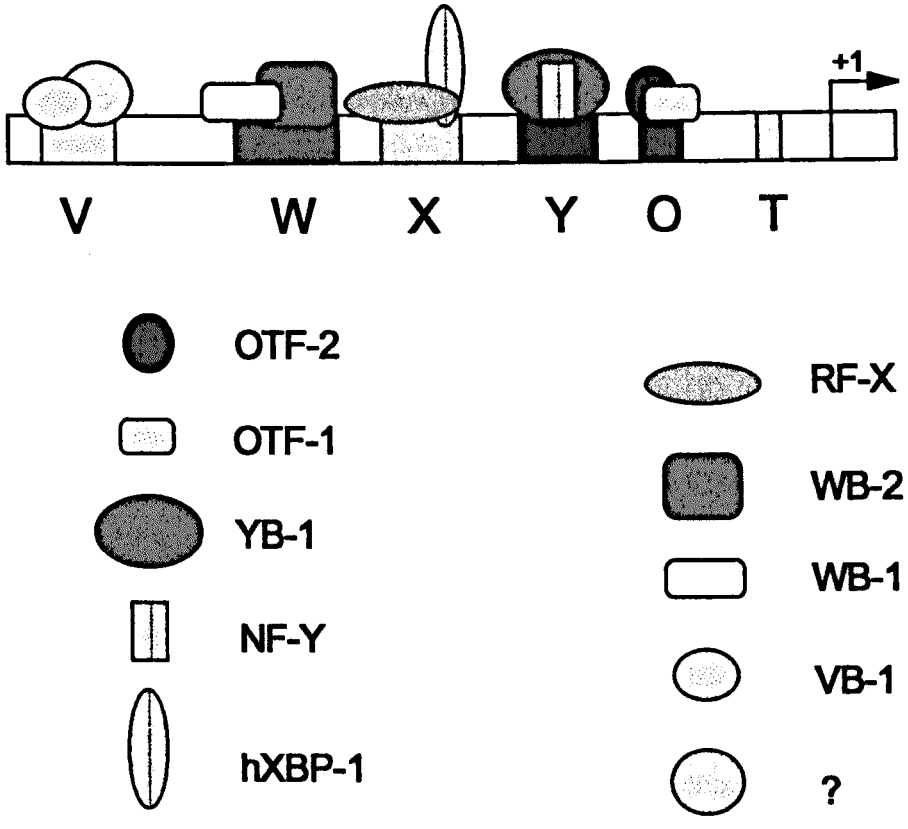
The octamer element is essential for HLA-DRα promoter function in DR^+ B and T cell lines, but not in other DR^+ cell lines (Tsang, *et al.,* (1989); Sherman, *et al.,* 1989; and Wright and Ting, 1992). This element binds the transcription factors OTF-1 and OTF-2.

vi) The TATA box

Although the TATA box does not have a rôle in the promoters of the murine class II MHC promoters Eα and Aα, its function in the transcription of HLA-DRα is now unequivocal (Matsushima, *et al.,* 1992). Therefore, in DRα, the TATA box serves its traditional rôle in fixing the transcriptional start site.

The transcription factors which have been shown to associate with the *cis*-acting elements of the HLA-DRα promoter are portrayed graphically in **figure 1.8.**

Figure 1.8. Interaction of transcription factors with the HLA-DRα promoter



Notes:

The binding of nuclear factors to the *cis*-acting elements of the HLA-DRα promoter has been assessed by bandshift and footprinting techniques. Transcription factors shown to associate with the promoter are given different shapes. A dotted line down the centre of a protein suggests that the protein is composed of more than one subunit. **Section 1.6.3.** discusses the individual functions of the *cis*-acting elements and their corresponding *trans*-acting factors.

1.6.4. IFN-γ-induction of transcription

Due to the nature of the expression of class II MHC antigens and their inducibility in a variety of non-haematopoietic tissues, the induction by IFN-γ has been extensively investigated. Unlike the promoters of class I MHC genes, which possess a discrete, defined interferon response element, IFN-γ-induction of class II MHC requires the cooperative action of all three sequence elements W, X and Y (Tsang, *et al,* 1990; Dedrick and Jones, 1990; Tsang, *et al,* 1988; Yang, *et al,* 1990; Moses, *et al.,* 1992; and Sloan, *et al.,* 1992). As well as the requirement for the presence of the three sequence elements, their stereospecific alignment within the promoter is also critical (Vilen, *et al.,* 1991; and Vilen, *et al.,* 1992). The IFN-γ signal was shown to involve protein kinase C in a promonocytic cell line (Gumina, *et al.,* 1990) in addition to Na^{2+} entry (Benveniste, *et al.,* 1991). The IFN-γ-induced expression of HLA-DRα and the other class II MHC genes has been shown to be transcriptional and dependent on *de novo* protein synthesis by its inhibition in the presence of cycloheximide (Amaldi, *et al.,* 1989; Kerr, *et al.,* 1990).

The IFN-γ-induction of HLA-DRα gene expression, therefore, represents a unique model of gene induction which involves a trimeric regulatory DNA moiety whose function is dependent on structural constraints. These constraints most likely reflect protein-protein interactions or unique DNA structures.

In human systems, the pursuit of an IFN-γ-induced factor which binds to

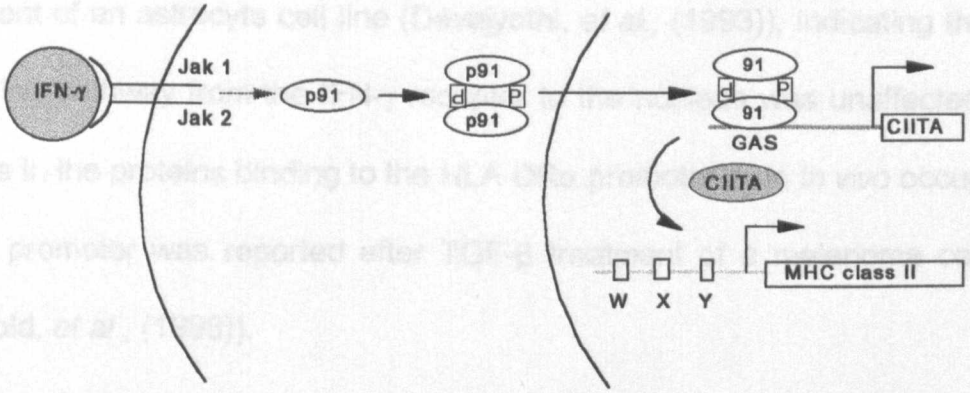
W, X or Y has been fruitless despite much research in many transformed cell lines. Conversely, when nuclear proteins from rat primary astrocytes were studied, a factor which was observed only in small amounts prior to IFN-γ-stimulation was very much enhanced post-stimulation (Moses, *et al.*, 1992). This factor, called IFNEX, was shown to associate consistently with the X box region, but was shown not to be serologically related to RF-X.

Recent hypotheses for the IFN-γ-induction of HLA-DRα transcription have focussed on the alteration of factors which are already bound to the promoter - IFN-γ stimulation leads to enhanced interaction of factors at the X and Y boxes. Uninducible cell lines are considered to have "open" X/X_2 and Y sites (Ting, 1993). The enhanced interaction of factors with the promoter is considered to be due to their modification or the increased accessibility of the promoter itself. The possibility of a promoter organising factor has been studied intensively with respect to combined immune deficiency (CID) by Glimcher *et al.*, who have employed *in vivo* footprinting techniques to determine the deficiency in this disease. The possibility for a class II promoter organising factor, which affects promoter accessibility with regards to transcription factors, is addressed in Kara and Glimcher, (1993).

A breakthrough in research into the IFN-γ-induced transcription of HLA-DRα came with the discovery of CIITA (class II transcriptional activator) (Steimle, *et al.*, 1994). This factor was shown here to be defective in one type of bare lymphocyte syndrome and as such is assumed to be essential for

constitutive MHC class II expression also. The gene for CIITA is induced by IFN-γ in a similar manner to that for the guanylate binding protein gene (GBP) which contains the first described γ-activated sequence. Induction of CIITA is thought be caused by the binding of active p91 to its promoter. This novel gene product appears, therefore, to be the much sought-after link between IFN-γ binding at the cell surface and the activation of class II MHC gene expression. There is no evidence for the direct association of CIITA with the MHC class II promoter and it is proposed that it may act as a coactivator which affects the transcriptional activity of proteins already bound to the promoter without binding itself. **Figure 1.9.** demonstrates the proposed mechanism for the action of IFN-γ in the induction of class II MHC gene expression.

Figure 1.9. Action of IFN-γ in the induction of class II MHC gene expression



The induction of class II MHC genes by IFN-γ involves the intermediate gene product CIITA (class II transactivator) which is synthesised *de novo* following IFN-γ-stimulation of the cell. p91 is the 91kD subunit of ISGF3 which becomes phosphorylated by Jak1 and Jak2 tyrosine kinases associated with the IFN-γ receptor. CIITA is believed to activate transcription of class II MHC genes without interacting with the promoter DNA itself (Steimle, *et al.*, (1994)).

1.6.5. Inhibition of the IFN-γ-induced expression of class II MHC by IFN-αβ and TGF-β

The IFN-γ-induced surface expression of class II MHC antigens has been reported to be affected by a cell's cotreatment with IFN-γ and IFN-αβ or IFN-γ and TGF-β (Morris and Tomkins, 1989; Darley, *et al.*, 1993). There has been little research in to the negative regulation of HLA-DRα transcription by these cytokines.

Devajyothi, *et al.*, (1993) and Reimold, *et al.*, (1993), simultaneously reported that 176bp of upstream sequence of the HLA-DRα promoter were required for TGF-β and IFN-αβ-mediated suppression of IFN-γ-induced reporter gene expression. It was also shown that IFN-γ-induced reporter gene expression from the ICAM-1 promoter was not affected by TGF-β or IFN-αβ treatment of an astrocyte cell line (Devajyothi, *et al.*, (1993)), indicating that the signalling pathway from the IFN-γ receptor to the nucleus was unaffected. No change in the proteins binding to the HLA-DRα promoter or in *in vivo* occupancy of the promoter was reported after TGF-β treatment of a melanoma cell line (Reimold, *et al.*, (1993)).

The influence of TGF-β on signal transduction mechanisms involved in IFN-γ-induced MHC class II gene expression was investigated in primary astrocytes (Panek, *et al.*, 1995). It was demonstrated that TGF-β inhibited IFN-γ-induced MHC class II surface protein, mRNA and promoter activity without affecting JAK1/2 and STAT 1α phosphorylation or IFNEX "induction". It

was postulated that, in astrocytes, TGF-β exerts its effects by modulation of the expression or function of constitutively expressed factors responsible for class II MHC gene expression.

The modulation of IFN-γ-induced class II gene expression by IFN-αβ has not been investigated as extensively and the involvement of both IFN-αβ and TGF-β in IFN-γ-induced class II gene expression in other cell systems has generally been reported at the level of the cell surface. The stage is therefore set for further research into the mechanisms of cytokine-mediated suppression of HLA-DRα induction.

1.7. Investigation objectives

As discussed in **section 1.2.2.** the induced expression of HLA-DRα by non-haematopoietic tissues may be of relevance not only in infection and inflammation, but also in conditions such as neoplastic disease and autoimmunity. The primary aim of this study was to examine a panel of human tumour cell lines which were either inducible or non-inducible for HLA-DR surface expression by IFN-γ. The study investigated the population of nuclear factors within these cell lines to determine whether HLA-DRα promoter-binding proteins were different from cell line to cell line. Also investigated was the stimulation of cells with IFN-γ to determine whether treatment had any effect on the populations of DNA binding proteins which were capable of binding to HLA-DRα promoter and to the individual regulatory elements.

A second aim was to study the effects of IFN-αβ and TGF-β upon the IFN-γ-induced expression of a reporter gene driven by HLA-DRα promoter sequences. The HLA-DRα promoter contains a consensus IFN-αβ response element from nucleotides -593 to -565 relative to the start of transcription, its involvement in any IFN-αβ-mediated suppression of induced expression was also to be investigated here.

CHAPTER 2

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell lines

The cell lines employed in this investigation were chosen to represent a range of MHC class II inducibility after interferon-γ treatment. The most common cell type was that which represented human colorectal epithelium, to furnish this requirement the cell lines chosen were: colo 201 (ATCC CCL 224; Semple, *et al.,* 1978), colo 205 (ATCC CCL 222; Semple, *et al.,* 1978), LS180 (ATCC CL 187; Tom, *et al.,* 1976), HT29 (ATCC HTB 38; Fogh and Trempe, 1975) and caco 2 (ATCC HTB 37; Fogh, 1977). All of these lines were originally derived from human colon adenocarcinomas.

The human glioblastoma cell lines U138MG and U373MG were also employed in certain studies due to their ease of manipulation.

The properties of these cell lines as regards expression of MHC antigens is shown in table 2.1.

2.2. Bacterial strains

The *E. coli* strain XL1-blue was used in all transformations and for the amplification and maintenance of plasmid vectors. A description of its genotype is given as follows: rec A1, end A1, gyr A96, thi-1, hsd R17, sup E44, rel A1, lac, [F' pro AB' $lacI^{9}Z\Delta M15$, Tn 10 (tet^{r})].

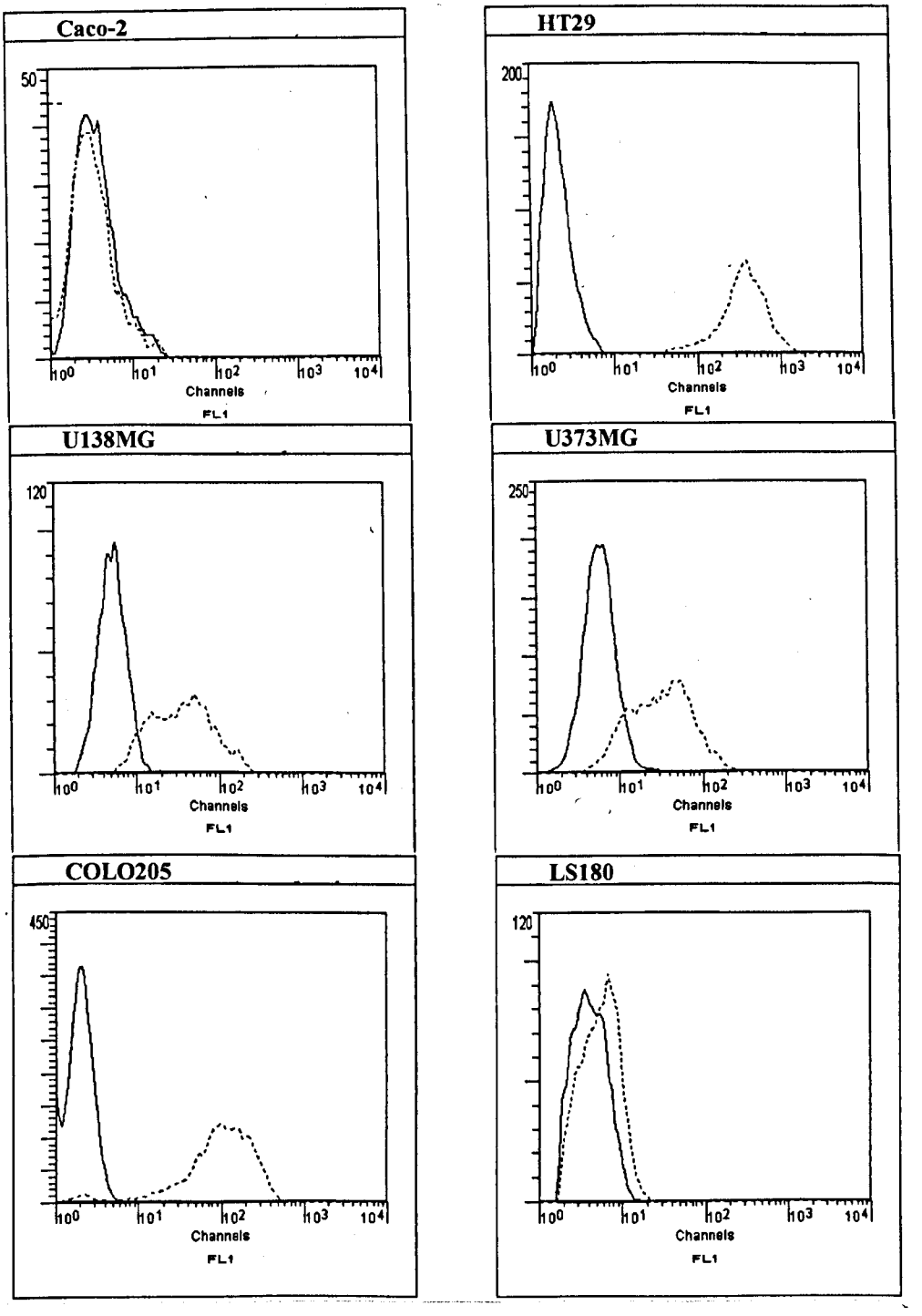
Table 2.1. Properties of cell lines

cell line	cell type	HLA-D inducibility*	HLA-D reduced by TGF-β?	growth inhibited by TGF-β?
colo 201 **colo 205**	colorectal	positive	no	yes
LS 180	colorectal	negative	-	yes
caco 2	colorectal	negative	-	-
HT29	colorectal	positive	no	-
U138MG	glioblastoma	positive	yes	stimulated
U373MG	glioblastoma (astrocytoma)	positive	yes	stimulated

* all cell lines were constitutively negative with respect to HLA-D expression. The inducibility of HLA-D at the cell surface refers to the expression of class II MHC after treatment of cells in culture with recombinant human interferon-γ for 48h.

The individual flow cytometric profiles for the induction of class II MHC (HLA-DR) in the cell lines indicated are shown in **figure 2.1.**

Figure 2.1. Induction of HLA-DR antigens by IFN-γ in cell lines employed



Cells were treated with 100U/ml IFN-γ for three days and stained for HLA-DR using FITC-labelled monoclonal antibody (clone L243, Becton Dickinson, Oxford, UK). An isotype-matched control antibody was included in all experiments (not shown) and was always indistinguishable from untreated cells stained for HLA-DR. Dashed lines indicate IFN-γ-treated cells; solid lines indicate untreated cells. Flow cytometric analysis was performed on a Becton Dickinson FACStar. Experiments performed by and histograms prepared by Dr. Wendy J. Bateman, University of Warwick.

2.3. Growth media

2.3.1. Tissue culture media

The colorectal cell lines colo 201 and colo 205 were grown in RPMI 1640 medium (Sigma chemical Co., Poole, Dorset, UK). All other cell lines were cultured in DMEM (Sigma). Medium was supplemented with 10% (v/v) foetal calf serum (Gibco), 2mM L-glutamine, and antibiotics; penicillin (100 iU/ml) and streptomycin (100 µg/ml). Medium supplemented with these additives is referred to as "complete medium".

2.3.2. Bacterial growth media

E. coli clones were grown in 2xYT broth supplemented with 100 µg/ml ampicillin (Sigma) to maintain plasmid vectors.

2xYT broth: 16g tryptone
10g yeast extract
5g NaCl
sterile distilled water to 1 litre

Medium was autoclaved prior to addition of ampicillin and growth of bacteria.

Solid medium was obtained by the addition of 1.5% (w/v) bacto-agar (Difco) prior to autoclaving at 121°C @ 20 psi pressure for 15 min. Indicator plates for the detection of *lac z* expression by recombinant colonies also contained 0.5mM IPTG and 0.02% (w/v) X-gal.

2.4. Oligonucleotides

Oligonucleotides were synthesised by the Applied Biosystems DNA synthesiser as single stranded molecules. Lyophilised oligonucleotides were typically suspended in 500µl sterile distilled water and the concentration of DNA calculated from measurement of OD_{260}. Stock oligonucleotides were aliquoted in 10µl volumes and stored at -20°C to prevent repeated freeze-thawing events.

Equimolar amounts of complementary strands were annealed by heating oligonucleotide mixtures at 90°C for 10min and allowing to cool for 3-4hr or overnight until the temperature attained that below the melting temperature (T_m) of each oligonucleotide. T_m was approximated by employing the formula:

$$T_m = 4(G+C) + 2(T+A)$$

Annealed oligonucleotides were diluted to 10pmol/µl, and stored at -20°C in 10µl aliquots.

The sequences of double-stranded oligonucleotides employed in protein-DNA binding analyses are given in table 2.2.

Table 2.2. Oligonucleotide sequences of double stranded DNA probes

PROBE NAME	RANGE IN DRα*	SEQUENCE
V BOX	-193 → -179	TGTTGTTGTT**GTTGTCCTGTTT**GTTTAGA ACAACAACAA**CAACAGGACAAA**CAAATCT
W BOX	-139 → -114	CGTGTCCT**GGACCC**TTTGCAAGAACCG GCACAGGA**CCTGGG**AAACGTTCTTGGC
X BOX	-110 → -85	CC**CCTAGCAACAGATG**CGTCATCTC GG**GGATCGTTGTCTAC**GCAGTAGAG
Y BOX	-84 → -61	AAAATATTTTT**CTGATTGGCC**AAAG TTTTATAAAAA**GACTAACCGG**TTTC

Notes:

* refers to the corresponding nucleotide positions of sequences in the promoter of HLA-DRα.

All sequences are given 5' to 3' with the coding strand above the non-coding strand.

Bold underlined text indicates the consensus protein binding sites.

2.5. Plasmids

2.5.1. pBS (KS$^+$) DRα 680 vectors

The HLA-DRα680 fragment

The polymerase chain reaction was employed to generate a fragment of DNA corresponding to the region of the HLA-DRα gene from nucleotides 511 to 1191 (the HLA-DRα680 fragment; numbering system from the Beckman Microgenie database and as cited in Das, *et al.*, 1984)). This fragment spanned the proximal promoter region and extended into the first transcribed nucleotides

of the HLA-DRα gene such that nucleotides -540 to +40 relative to the cap site were included. The cloning of this fragment into the phagemid pBluescript (KS^+) allowed further manipulation (by PCR and restriction endonuclease digestion) and subcloning into other plasmid vector types. Preparation and subsequent cloning of this fragment was performed by Mr. George Ward (Biological Sciences, University of Warwick). The sequences of primers employed in PCR amplification of HLA-DRα fragments are given in **table 2.3.** The method by which the DRα680 fragment was amplified is outlined below in **table 2.4.**

Table 2.3. Oligonucleotide primers employed in the PCR amplification of HLA-DRα promoter fragments

PRIMER NAME	SEQUENCE (5'→3')	REGION BOUND IN HLA-DRα
DR1	GGATCGAGTCAGTAGAGCTCGGGAG	+22→+42
DR2	CCTGTGTTTCAAGATACAGCCGATCC	-641→-621
DR4	GGATCCTCTTTGGCCAATCA	-73→-60

The above table gives the sequences of the single stranded DNA oligonucleotide primers (DR1, DR2 and DR4) employed in the generation of the HLA-DRα promoter fragments DRα680 and DRα582. Combinations of DR2 with DR1 and DR4 in PCR reactions were used to amplify 680 and 582 base pair fragments, respectively. The regions to which the primers bind within the HLA-DRα gene are indicated in relation to the start of transcription (cap site). **Bold** sequences indicate the presence of an introduced restriction endonuclease recognition sequence.

Table 2.4. Summary of the reaction conditions employed for the amplification of the HLA-DRα 680 promoter fragment

COMPONENT	ATTRIBUTES
target DNA (genomic)	1µg
PCR primers, DR1 & DR2	2.5pM
deoxy nucleoside triphosphates	200µM
magnesium^{2+}	1.5mM
Taq DNA polymerase	2.5U
denaturation annealing/elongation	94°C, 1min 72°C, 1.5 min } 30 cycles
final elongation	72°C, 10 min

This table describes the components and conditions for the polymerase chain reaction amplification of the 680 base pair fragment of the HLA-DRα promoter. The sequences of the primers DR1 and DR2 and the regions of the HLA-DRα gene with which they associate are defined in **table 2.3**.

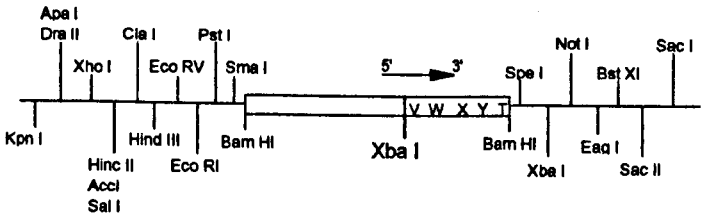
2.5.2. Preparation of the pBluescript(KS$^+$)DRα680 vectors

These vectors were prepared by the appropriate restriction endonuclease digestion of the PCR-generated HLA-DRα680 fragment and the phagemid vector pBluescript and the subsequent enzymatic ligation of the two moieties. Generation of cohesive ends in the HLA-DRα680 fragment was achieved by digestion with Bam HI - this was made possible by the introduction of artificial restriction sites into the primers for the PCR reaction. The pBS(KS$^+$)DRα680 vectors were prepared by Mr. George Ward, Department of Biological Sciences, University of Warwick.

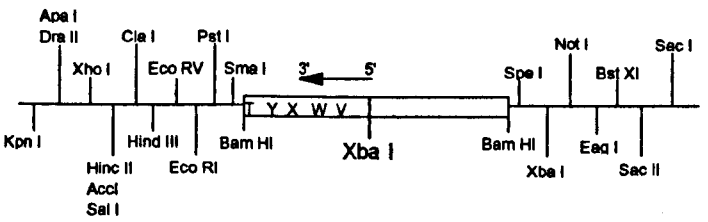
This cloning procedure yielded two vectors which contained the HLA-DRα680 promoter fragment in 5'→3' or 3'→5' orientation relative to the pBluescript(KS$^+$) polylinker. Fragment orientation was determined by the characteristic sizes of restriction fragments obtained by digestion of recombinants with Xba I. These vectors were named pBS(KS$^+$)DRα680 5'→3' and pBS(KS$^+$)DRα680 3'→5'. The orientation of the HLA-DRα680 promoter fragment relative to the pBluescript polylinker in these vectors is represented schematically in **figure 2.2.**

Figure 2.2. Orientations of the HLA-DRα680 promoter fragment in the pBS(KS$^+$)DRα680 vectors

a) pBS(KS$^+$)DRα680 5'→3'



b) pBS(KS$^+$)DRα680 3'→5'



Notes:
Figures **(a)** and **(b)** represent the two vectors obtained from the cloning of the PCR-generated 680 base pair HLA-DRα promoter fragment into Bam HI-linearised pBluescript (KS$^+$). Nomenclature for the recombinants was dependent upon the orientation of the cloned promoter fragment with respect to that of the polylinker of the vector.

2.5.3. Preparation of HLA-DRα CAT expression vectors

pDRα680CAT

Chloramphenicol acetyl transferase vectors were employed in a variety of reporter gene studies devised to elucidate the possible mechanisms of cytokine-mediated control of HLA-DRα gene expression. Vectors were prepared by the cloning of DNA fragments corresponding to regions of the HLA-DRα promoter into the multiple cloning site of the eukaryotic expression vector pCATbasic (Promega) such that a "functional" promoter was provided for the expression of chloramphenicol acetyl transferase from this plasmid following transient transfection and appropriate cytokine treatment of cells. The pCATbasic vector itself lacks eukaryotic promoter and enhancer sequences, but provides insertion points for the cloning of promoter or enhancer elements (see **figure 2.3.**). Amplification of pCATbasic (and derivatives thereof) was permitted by the presence of a bacterial origin of replication and a β-lactamase coding region for selective growth in transformed *E. coli* in the presence of ampicillin. All CAT plasmids contained SV40 donor and acceptor splice sites and polyadenylation signals form the gene encoding the small t-antigen.

The vector pDRα680CAT contained the 680 base pair HLA-DRα promoter fragment upstream of the structural CAT gene of pCATbasic. Cloning of this promoter fragment into this vector was achieved in the following manner: pCAT basic was linearised by restriction endonuclease digestion with Sal I and the cohesive ends blunted by the Klenow reaction. A second

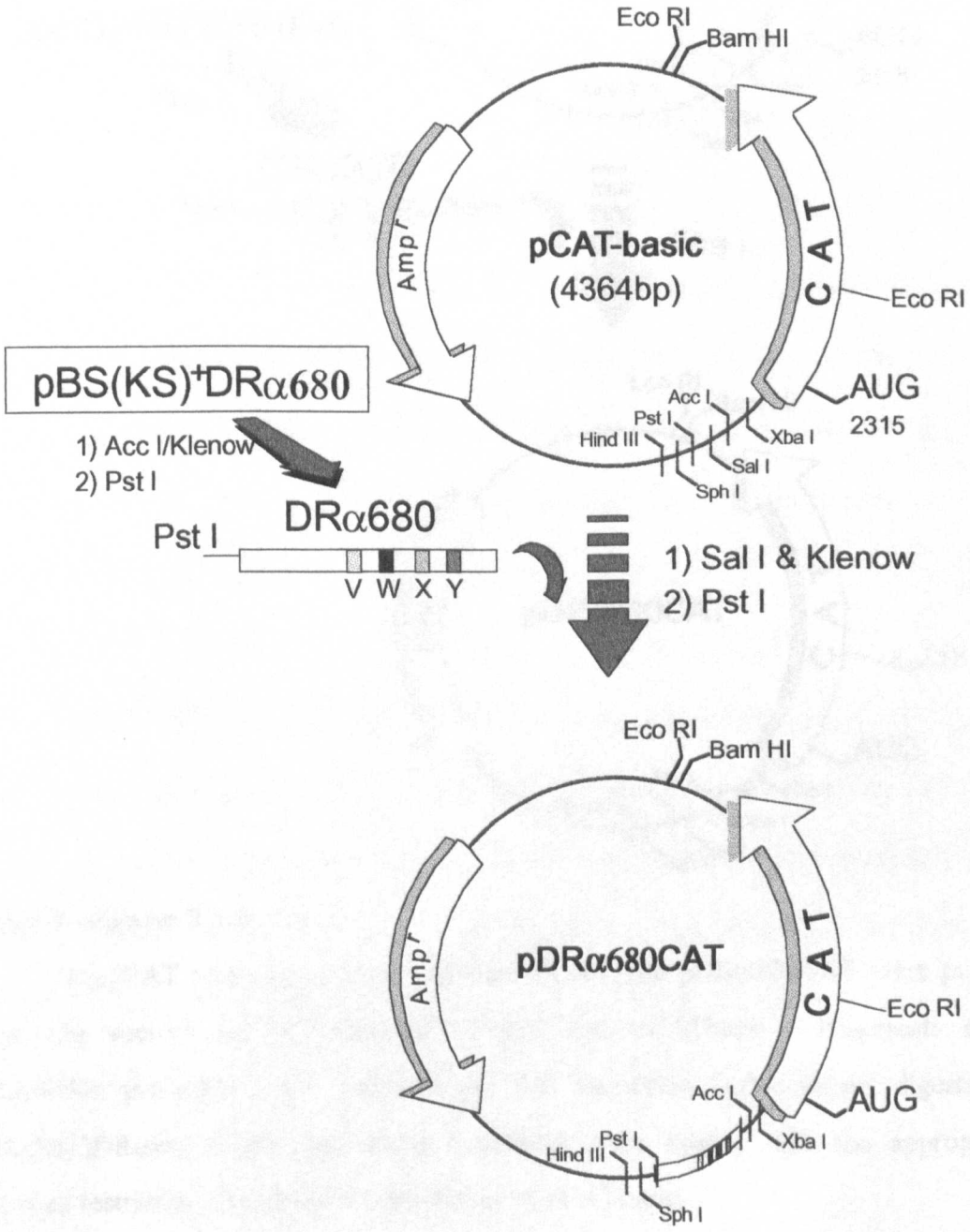
digestion with Pst I yielded a Pst I-blunt site for the ligation of a HLA-DRα680 fragment generated by the a) Acc I digestion followed by Klenow end-filling, then b) Pst I digestion of pBS(KS$^+$)DRα680 5'→3'. Ligation yielded a recombinant vector in which the HLA-DRα promoter fragment had been inserted in the correct orientation for transcription of the CAT gene. This cloning strategy is summarised in **figure 2.3.**

pDRα320CAT

A deletion mutant of the HLA-DRα promoter was prepared such that the effects of loss of the upstream region of the promoter could be assessed. This deleted promoter fragment, termed DRα320, was again cloned into the polylinker of pCATbasic. Preparation of the vector was achieved by its restriction endonuclease digestion with Xba I and dephosphorylation by treatment with calf intestine alkaline phosphatase as described. The DRα320 promoter fragment was prepared by "dropping out" the appropriate piece of DNA from pBS(KS$^+$)DRα680 5'→3' by its digestion with XbaI. The DRα320 fragment was purified by electrophoresis through agarose, followed by electroelution and subsequent purification by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (detailed in section **2.8.**). Ligation was performed "blind" such that the recombinants may have contained this promoter fragment in either orientation with respect to the CAT structural gene. Those recombinants with the promoter fragment in its correct orientation were identified by restriction endonuclease digestion with Spe I and Hind III - this

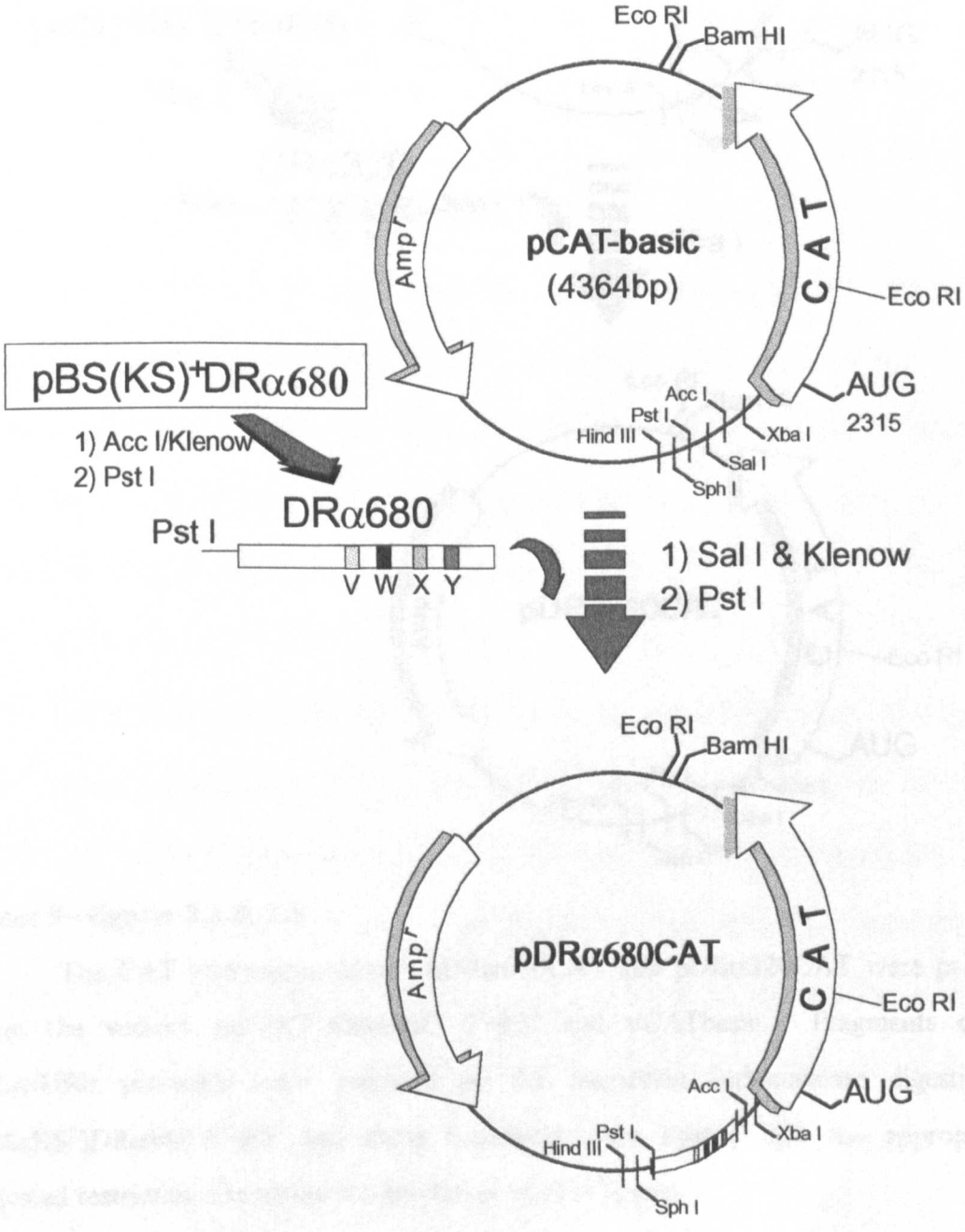
digestion released a 344bp fragment from those recombinants with the 320 bpHLA-DRα promoter fragment in the correct orientation. The cloning strategy employed for the preparation of pDRα320CAT is represented schematically in **figure 2.4.**

Figure 2.3. Cloning strategy for the preparation of the pDRα680CAT expression vector



digestion released a 344bp fragment from those recombinants with the 320 bpHLA-DRα promoter fragment in the correct orientation. The cloning strategy employed for the preparation of pDRα320CAT is represented schematically in **figure 2.4.**

Figure 2.3. Cloning strategy for the preparation of the pDRα680CAT expression vector



2.5.4. pSV2CAT and p0CAT

The plasmid pSV2CAT contained the SV40 late promoter to drive the expression of the CAT gene. Plasmid p0CAT contained only the CAT reporter gene with no promoter sequence to drive gene expression. This vector was prepared by the removal of the SV40 late promoter sequence from pSV2CAT by its restriction endonuclease digestion with Accl and Hind III followed by its religation after formation of blunt ends. These plasmids were prepared by Mr. George Ward; schematic representations are given in **figures 2.5 & 2.6.**

2.5.5. The β-galactosidase expression vector

Plasmid pRSVβ-gal expressed the bacterial *lac z* gene (β-galactosidase) from the Rous Sarcoma virus long terminal repeat (LTR). Polyadenylation and small t-antigen donor and acceptor splice sites for the processing of transcripts were from SV40. This vector also contained a bacterial origin of replication and β-lactamase gene for propagation in transformed *E.coli.*

This vector was prepared by Dr. Simon Swingler, University of Warwick, (PhD thesis, 1992) by the ligation of a 3736 fragment containing the *lac z* open reading frame (generated by the Hind III-Bam HI restriction digestion of plasmid pCH110 (Pharmacia)) into Hind III-Bgl II digested RSV-33. RSV-33 is a eukaryotic expression vector which contains the RSV LTR, SV40 processing signals and sequences for maintenance and propagation in bacteria. A diagram of pRSVβ-gal is shown in **figure 2.7.**

Figure 2.5. pSV2CAT

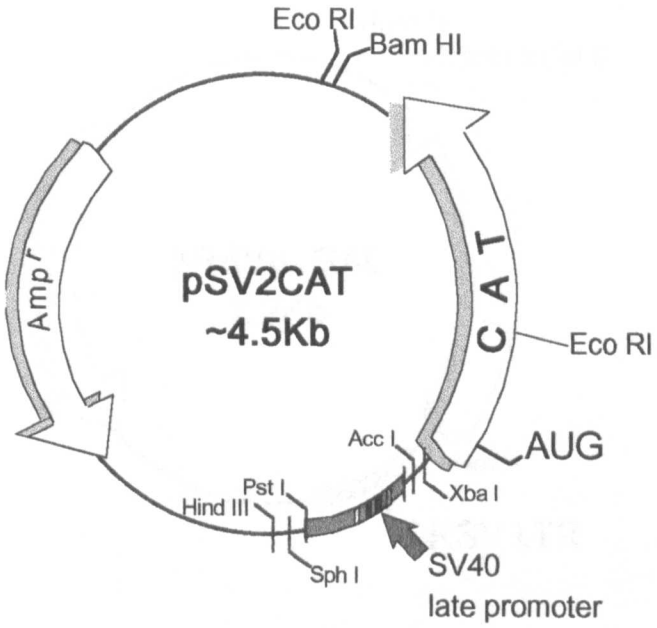
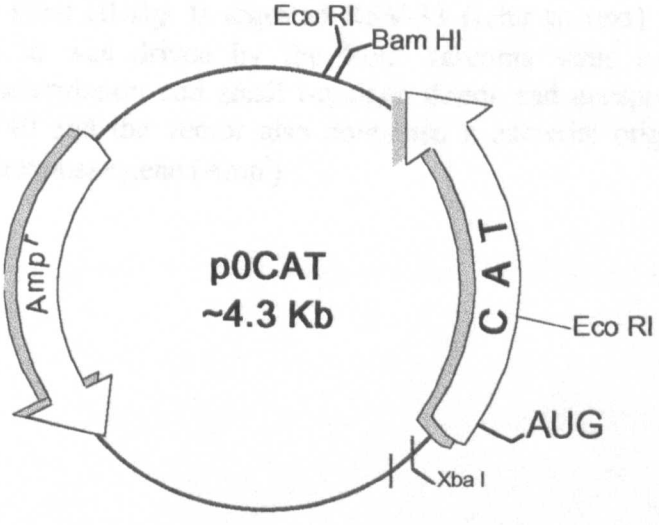
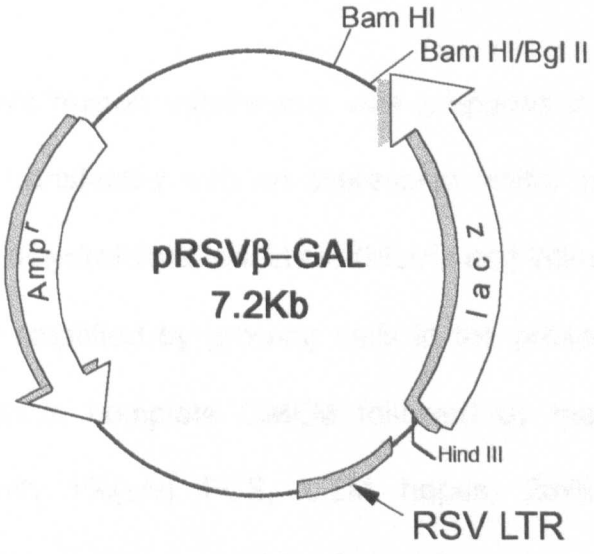


Figure 2.6. p0CAT



Figures 2.5& 2.6: pSV2CAT was a positive control vector for the transfection of eukaryotic cells and transfected cells' ability to process reporter gene transcripts. Transcription of the chloramphenicol acetyl transferase gene was directed by the SV40 late promoter. This vector also contained the gene for β-lactamase (Amp^r) in addition to SV40 polyadenylation and splice sites for the processing of CAT transcripts (not shown). Removal of the SV40 late promoter by restriction endonuclease digestion with Hind II and Acc I, followed by ligation of Klenow-generated blunt ends resulted in the negative control vector p0CAT.

Figure 2.7. pRSVβ-gal



The eukaryotic expression vector pRSVβ-gal contained the bacterial *lac z* gene, which encodes β-galactosidase, and was employed in transient transfection studies as a positive control. It was prepared by the ligation of a 3736bp fragment containing the *lac z* sequence into the Hind III-Bgl II digested RSV-33 (refer to text). Expression of the β-galactosidase gene was driven by the Rous sarcoma virus long terminal repeat (RSV LTR). Polyadenylation and small t-antigen donor and acceptor splice sites were included from SV40 and the vector also contained a bacterial origin of replication in addition to the β-lactamase gene (Amp^r).

2.6. Cytokines

Recombinant human interferon-γ was prepared from CHO-DHFR$^-$cells (clone 12CMTX) transfected with an expression vector containing cDNAs for human IFN-γ and dihydrofolate reductase (Morris and Ward, 1987). Expression of the IFN-γ was amplified by growing cells in the presence of methotrexate. Cells were grown in complete GMEM followed by maintenance in αMEM supplemented with 1%(v/v) FCS, 0.2M hepes, 2mM L-glutamine, 0.1M Na_2HCO_3, 100iU/l penicillin and 100μg/l streptomycin. Maintenance medium was harvested daily, filtered and supplemented with 0.02% sodium azide. Interferon-γ was partially purified by gel filtration through a blue sepharose column, collected in 1ml aliquots and stored at -70°C. The specific activity was assessed by Semliki Forest Virus assay (performed by AGM).

Human IFN-αβ and TGF-β_1 were purchased from Sigma and British Biotechnology Products, Ltd., respectively.

2.7. Cell culture

2.7.1. Growth of cell lines in culture

Suspension cells were grown in complete RPMI 1640 medium in tissue culture grade plastic flasks (Costar) at 37°C in a humidified atmosphere of 5% CO_2. The cells were maintained at an approximate density of 10^5 cells/ml by dilution in fresh, prewarmed medium every 3-4 days.

Adherent cells were grown in complete DMEM in the conditions specified and the medium changed every 2-3 days. Upon confluence, the monolayers were washed briefly with warm PBS then incubated with 2-5ml trypsin-EDTA solution (5mg/ml trypsin, 5mM EDTA; Gibco) at room temperature until the cells detached from the inner surface of the flask. The cell suspension was added to an equal volume of prewarmed complete medium and the cells pelleted by centrifugation at 400*g* for 5min. The pellet was resuspended in an appropriate volume of complete medium and the cells reseeded into fresh medium at a dilution of 1:8 to 1:10 in a new flask.

2.7.2. Storage of cells in liquid nitrogen

Confluent monolayers were trypsinised and pelleted as described. The cells recovered from a $25cm^2$ surface area were resuspended in 1ml of freezing medium (95% FCS, 5% DMSO) and allowed to slowly freeze to -70°C for 24hr. The cells were then transferred to liquid nitrogen for long term storage.

2.7.3. Recovery of cells from liquid nitrogen

Vials were carefully removed from liquid nitrogen and placed in a waterbath at 37°C to facilitate rapid thawing of the cell suspension. The cells were carefully diluted into 10ml complete DMEM then pelleted by centrifugation to remove DMSO. The pellet was then resuspended in 5ml complete medium and a 25cm² flask seeded with the cell suspension. The medium was replenished daily until the cells reached confluence.

2.8. Routine manipulation of DNA

2.8.1. Phenol chloroform extraction

Contaminants, e.g. protein, were removed from solutions containing DNA by adding an equal volume of phenol / chloroform / isoamyl alcohol (25:24:1) and vortexing. The phases were separated by centrifugation at 20,000*g* for 5-10min. The aqueous (top) layer was recovered (such that precipitated protein at the interface was not disturbed) and transferred to a fresh tube. Occasionally, it was necessary to "back-extract" the solvent phase. This was achieved by adding an equal volume of distilled water (or other aqueous reaction solution) to the phenol, vortexing and centrifuging as described. The aqueous phases (containing DNA) were combined and either extracted again with phenol or the DNA precipitated with alcohol as described below.

2.8.2. Precipitation of DNA

The concentration of sodium acetate, pH 5.2 (or sodium chloride) in aqueous DNA solutions was adjusted to 0.3M. Two and a half volumes of 100% ethanol, or an equal volume of isopropanol, were added and the mixture vortexed. Precipitation was allowed to occur at -20°C overnight, -70°C for 30-60min, or in a dry ice/ethanol bath for 15-20min. Recovery of DNA was performed by centrifugation at 20,000*g* for 10-20min. Pellets were washed twice with 70% ethanol to remove salt and allowed to air-dry, or dried briefly under vacuum, prior to resuspending the DNA in TE buffer (10mM Tris-HCl, pH 7.6; 1mM EDTA) or sterile distilled water.

2.8.3. Gel electrophoresis of DNA

i) Agarose gel electrophoresis.

Routine analysis of DNA was performed by horizontal electrophoresis through agarose gels buffered with 1xTBE (89mM Tris-HCl, pH 8.3, 89mM boric acid, 10mM EDTA) containing 0.5μg/ml ethidium bromide. Electrophoresis was performed in a Gibco-BRL model H5 horizontal gel tank and agarose gels were poured into 11.0cmx14.0cm plastic mould. Wells were formed by using 1 or 2mm thick plastic combs of varying numbers of teeth depending on sample volume and number of samples. Prior to loading, samples were mixed with one fifth volume load dye (5xTBE, 30% [v/v] glycerol, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol). Electrophoresis was performed at 60-120V at room temperature. Size markers (Gibco-BRL 1Kb ladder with fragment sizes ranging

from 0.1-12Kb) were also electrophoresed alongside samples to provide a reference for identification of characteristically-sized DNA fragments within the samples. Upon completion of electrophoresis, the DNA in the gel was visualised under U/V light and photographed using a Polaroid camera and type 55 4"x5" land film.

ii) Electro-elution of DNA from agarose gels.

Purification of electrophoresed DNA within agarose was performed by the electro-elution method described by Sambrook *et al.* (1989). The piece of agarose containing the required piece of DNA was excised from the whole gel using a scalpel blade. The gel slice was then placed into a dialysis bag containing 0.5ml 1xTBE and the bag sealed with a clip. This was then submerged in the electrophoresis buffer in the gel tank and electrophoresis performed at 100 V for 30 min. After this time, the direction of current was reversed for 30sec to liberate any DNA from the dialysis tubing itself. The dialysis assembly was then visualised under U/V to confirm the extraction of DNA from the gel into the buffer. The buffer containing the DNA was transferred from the dialysis tubing to a microcentrifuge tube and the tubing rinsed with 0.5 volumes TBE. The pooled buffer samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA precipitated with ethanol as described.

iii) Polyacrylamide gel electrophoresis.

Non-denaturing polyacrylamide gel electrophoresis of DNA was employed in the purification of end-labelled probes and in the separation of nucleoprotein-DNA complexes from free probe in bandshift experiments. Five percent (w/v) polyacrylamide gels were prepared from a 40% stock solution of acrylamide/bis-acrylamide (29:1) and were formed in a 150x170x1.5 mm (height x width x thickness) apparatus. Polymerisation was catalysed by the addition of 0.1% (w/v) ammonium persulphate and 0.1% (v/v) TEMED and was allowed to proceed for 1hr before gels were used. The ionic strength of gels was modified according to their requirements; gels being employed for preparation of radioactively labelled probes were typically of high ionic strength and contained 1xTBE, those being used in bandshift experiments were of low ionic strength and contained 0.2xTBE. Electrophoresis was performed in a Gibco-BRL model V15.17 vertical gel tank at 150V in 1xTBE or 0.2xTBE for low ionic strength gels.

iv) Extraction of DNA from polyacrylamide gels.

Radiolabelled probes were localised by autoradiography against Fuji RX100 for 1min. The piece of gel containing the probe was excised with a clean scalpel blade and cut into 1mm cubes. The DNA was eluted from the gel by the "crush and soak" method (Sambrook *et al.*, 1989): an equal volume of elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA [pH 8.0], 0.1% (w/v) SDS) was added to the crushed gel slice in a microcentrifuge tube and shaken overnight on a rotating platform to elute the

DNA. The sample was vortexed, centrifuged briefly and the supernatant centrifuged through siliconised glass wool at 500*g* for 5min to remove any remaining polyacrylamide: a 1ml syringe was packed with glass wool to ≈2cm height and placed above the open neck of a 1.5ml microcentrifuge tube inserted into a 15ml Corex tube. The supernatant, collected in the microcentrifuge tube after the centrifugation step, was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol and the DNA precipitated with 100% ethanol. After washing with 70% ethanol and resuspending in TE or sterile distilled water, the DNA was reprecipitated and washed twice further with 70% ethanol prior to air-drying. Samples were resuspended in an appropriate volume of TE or sterile distilled water (typically 200μl) and stored at -20°C until use.

2.8.4. Use of DNA modifying enzymes

i) Restriction endonuclease digestions.

Restriction enzymes were typically supplied by Gibco-BRL or Amersham and were provided with manufacturers' reaction buffer; all reagents were kept on ice during the preparation of incubations. The DNA to be cleaved was prepared in sterile distilled water and the appropriate volume of 10 x reaction buffer in a microcentrifuge tube. The number of units of enzyme required to perform the digestion in the given time (1-24hr) was calculated from the concentration of the enzyme, the frequency of cleavage of bacteriophage λ DNA for the particular enzyme and the amount (in μgs) of DNA to be digested. The required amount of enzyme was added to the DNA/reaction buffer mixture

to start the reaction and the tube incubated at the appropriate temperature for the required length of time.

ii) Dephosphorylation of DNA.

Prior to using digested DNA in further reactions, it was often necessary to dephosphorylate the 5' ends. This was achieved by the use of calf intestine alkaline phosphatase (CIAP). Following restriction enzyme digestion, the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol and ethanol precipitated. The pellet was resuspended in 50µl CIAP buffer (10mM Tris-HCl [pH8.3], 1mM $ZnCl_2$, 1mM $MgCl_2$), for 5' overhanging ends, 1U CIAP/100pmoles DNA was added, whereas for blunt ends or recessed termini, 1U/2pmoles DNA was added. Reaction mixtures were incubated at 37°C for 30min, after which time a further aliquot of CIAP was added and tubes were incubated for a further 30min at 45°C.

iii) Blunt end reaction.

The Klenow fragment of *E. coli* DNA polymerase was used in the presence of deoxynucleoside triphosphates to generate blunt-ended DNA molecules from those possessing 5' overhangs created by restriction endonuclease digestion. Purified restricted DNA was resuspended in buffer (0.5M Tris-HCl [pH 7.5], 0.1M $MgSO_4$, 1mM DTT, 500µg/ml bovine serum albumin [fraction V]) containing 1mM dNTPs. The reaction was started by adding 1 unit of enzyme per microgram of DNA and allowed to proceed by incubating at room temperature for 30min. Reactions were terminated by the

addition of 1μl 0.5M EDTA (pH 8.0) followed by phenol chloroform extraction and ethanol precipitation.

iv) Ligation.

The procedure for ligating blunt and cohesive ends was identical. In a final volume of 50μl ligase buffer (50mM Tris-HCl [pH 7.6], 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000), 100μg of dephosphorylated vector DNA was mixed with an equimolar amount of insert DNA possessing compatible termini. One unit of T4 DNA ligase was added and the mixture incubated at 16°C for 18hr. Following ligation, samples were stored at -20°C until use.

2.8.5. Transformation of bacteria and preparation of plasmid DNA

i) Preparation of competent bacteria.

Transformation-competent bacterial cells were prepared from the method of Sambrook, *et al.*, (1989). A 10ml culture of XL1-blue cells in 2xYT broth containing 15μg/ml tetracycline was grown overnight at 37°C with gentle shaking. The next day, 1ml of the resulting cell suspension was subcultured into 100ml 2xYT broth containing tetracycline and grown for a further 3hr until the culture had reached OD_{600} of approximately 0.3. The culture was then cooled on ice for 15min and the cells pelleted by centrifugation at 1500*g* for 10min at 4°C. The cells were resuspended in 10ml of 100mM $MgCl_2$ and incubated on ice for 10min prior to repelleting by a further centrifugation step. After resuspending in 5ml of 100mM $CaCl_2$ containing 15% (v/v) glycerol, the

cells were aliquoted into 100µl volumes and either incubated at 4°C for an hour prior to transformation or stored at -70°C until use.

ii) Transformation of competent bacteria with plasmid vectors.

Twenty five microlitres of a ligation reaction mixture (or 1-5µl (1-5µg) of amplified plasmid DNA) were incubated in a with 100µl competent cells in a 1.5ml microcentrifuge tube for 1hr on ice. The mixture was then heated at 42°C for 2min prior to a further incubation on ice for 30min. Antibiotic-free 2xYT broth (900µl) was then added and the mixture incubated at 37°C for 30min. Fifty microlitres of the cell suspension were plated out onto 2xYT agar containing ampicillin (and IPTG & X-gal). The remaining cells were then pelleted by centrifugation at 20,000*g* for 1min in a microcentrifuge, the supernatant was discarded and the pellet resuspended and plated out onto a fresh 2xYT agar plate. Plates were incubated overnight at 37°C. All vectors employed contained the gene conferring ampicillin resistance and plasmid constructs based on pUC and pBluescript II carried the β-galactosidase gene whose expression was inactivated by the presence of an insert. Recombinants were identified by their inability to metabolise the chromogenic galactose analogue X-gal in solid media.

iii) Small scale preparation of plasmid DNA.

The "TENS" buffer mini-prep method was used to isolate plasmid DNA from bacterial colonies prior to its characterisation by restriction endonuclease digestion.

Single colonies were inoculated into 5-10ml 2xYT broth containing ampicillin and grown overnight at 37°C in an orbital shaker. The following day, 1.5ml of cell suspension was transferred to a microcentrifuge tube and the cells pelleted by centrifugation at 20,000*g* for 1min. The supernatant was decanted off leaving 50-100µl in the tube and the cells were resuspended by vortexing. Three hundred microlitres of ice-cold TENS buffer (10mM Tris-HCl, [pH 8.0], 1mM EDTA, 0.1 M NaOH, 0.5M sodium dodecyl sulphate) were added and the tubes vortexed for 2-5sec. After the addition of 150µl 3M sodium acetate, the tubes were vortexed again and the resultant cell debris was pelleted by centrifugation at 20,000*g* for 2min. The supernatant was transferred to a clean microcentrifuge tube and 900µl of 100% ethanol which had been pre-cooled to -20°C was added. Tubes were centrifuged for 5min at 20,000*g* to pellet the precipitated plasmid DNA. Pellets were washed with 1ml 70% ethanol and dried under vacuum. The DNA was dissolved in 40µl sterile distilled water containing 50µg/ml RNase A. Five microlitres of the resulting plasmid solution were used for restriction enzyme analysis.

Upon identifying clones of recombinant bacteria containing the desired plasmid, glycerol stocks of cells were prepared for long term storage. This was achieved by pelleting 1.5ml of bacterial cell suspension and resuspending the cells in 2xYT broth containing 40% (v/v) glycerol. Glycerol stocks of bacterial clones were stored at -20°C for up to 12 months.

iv) Large-scale preparation of plasmid DNA.

Milligram quantities of plasmid DNA were prepared by the method of Sambrook, *et al.*, (1989). An overnight culture of bacteria was prepared by the inoculation of 10ml 2xYT broth containing 0.1mg/ml ampicillin with 10µl of cells from a glycerol stock (or 100µl of a 4°C cell stock) and cultures were grown at 37°C with shaking. One to five millilitres of the resulting culture were used to inoculate 500ml of 2xYT broth containing ampicillin and the cells were allowed to grow overnight at 37°C in an orbital shaker. Pelleting of the cells was achieved by centrifugation (Beckman J14, 7,000rpm for 5min) and supernatants were discarded. Cell pellets were resuspended in 10ml solution I (50mM glucose, 25mM Tris-HCl [pH 8.0], 10mM EDTA), transferred to a 50ml Falcon tube and the bacteria lysed by the addition of 20ml freshly made solution II (0.2M NaOH, 1% [w/v] SDS): following addition of solution II, the suspension was gently mixed by inversion and the tubes allowed to stand on ice for 5-10min. After this time, 15ml ice-cold solution III (3M potassium acetate, 3M acetic acid) was added, the tubes mixed by inversion and allowed to stand on ice for 10min. Bacterial cell debris and DNA was then removed by centrifugation at 1800*g* for 30min at 4°C in an MSE Chilspin 2 (4,100rpm). Supernatants were transferred to clean Falcon tubes containing 0.6 times their volume of isopropanol and the DNA was allowed to precipitate at room temperature for 15-20min. The precipitated DNA was pelleted by centrifugation at 1800*g* for 30min, following which it was washed twice with 70% ethanol, air-dried, then resuspended in 7.5ml TE prior to purification of the plasmid DNA by caesium chloride density gradient.

v) Purification of plasmid DNA through caesium chloride density gradients.

A solution of caesium chloride was prepared by dissolving 28g CsCl in 20ml TE and was transferred to a Beckman "quick-seal" centrifuge tube and tubes were carefully balanced at this stage by the addition/removal of CsCl. The plasmid DNA solution was mixed with 0.5ml of 10mg/ml ethidium bromide and added to the TE in the Beckman tube and the tubes were rebalanced. The aqueous solution was overlaid with liquid paraffin and the tubes were heat-sealed at the neck. Tubes were then transferred to a Beckman VTi 50 rotor and centrifuged at 45,000rpm for 16hr; after which time, the centrifuge was allowed stop without brake. The bands of plasmid and genomic DNA were visualised under U/V light. The vacuum in the tube was released by piercing its top with a syringe needle and, following this, the lower (plasmid) band was recovered using a 10ml plastic syringe. Ethidium bromide was removed from the plasmid solution by extracting five times with an equal volume of H_2O-saturated butan-1-ol. The caesium chloride was then removed by dialysis versus three changes of 5L of TE; each dialysis step was allowed to proceed for 1h at room temperature. DNA was recovered by ethanol precipitation; after precipitating, washing and drying, the plasmid pellet was resuspended in 0.5ml TE or sterile distilled water and the concentration of DNA determined by measuring $OD_{260/280}$ at a dilution of 1:20. At 260nm, a 50μg/ml solution of dsDNA has an optical density of 1, and the purity of a DNA solution can be assessed by the ratio of ODs at 260 and 280nm - for pure DNA, this ratio is 1.8. In cases where this ratio was low, i.e. too much protein in the sample, the DNA was extracted with phenol/chloroform/isoamyl alcohol and reprecipitated.

2.9. Transfection of eukaryotic cells

2.9.1. Transfection of adherent cells.

Adherent cells were transfected with calcium phosphate / DNA coprecipitates prepared by a method modified from Gorman, *et al.*, (1985) and Chen and Okayama (1987). Twenty four hours prior to transfection, 60mm tissue culture dishes (Costar) were seeded with a sufficient number of cells ($3.5\text{-}6.5 \times 10^5$ cells/dish depending on cell type), prepared by the trypsinisation of confluent layers, to achieve 60-80% confluence the next day. Cells were grown in 5ml complete DMEM in an atmosphere of 5% CO_2 at 37°C overnight (20-24hr). The following day, 1ml of calcium phosphate/DNA precipitate was prepared for each pair of dishes. Forty micrograms (20μg per dish) of caesium chloride gradient-purified plasmid DNA was mixed with 50μl 2.5M $CaCl_2$ and the volume increased to 500μl with tissue culture grade sterile distilled water. An equal volume of 2xHBS (50mM Hepes-NaOH [pH7.12], 280mM NaCl, 10mM KCl, 12mM glucose, 3mM Na_2HPO_4) was then added dropwise with gentle shaking and the mixture allowed to stand at room temperature for 10-15min. Following this incubation, a fine coprecipitate of $Ca_3(PO_4)_2$-DNA could be observed. Precipitates were mixed gently by pipetting once and 500μl were added dropwise to the medium above monolayers on duplicate dishes with gentle swirling. Dishes were incubated at 37°C for 20hr. The next day, the medium containing the precipitates was removed and the monolayers were washed gently with 5ml of warm PBS. The cells were then glycerol shocked for 1min with 5ml 20% (v/v) glycerol (Sigma, tissue culture grade) in PBS, washed

twice with PBS and incubated at 37°C in 5ml complete DMEM. For transient transfection studies, the dishes were incubated until fresh medium had equilibrated with the temperature and CO_2 atmosphere (approximately 1hr) before the dropwise addition of cytokines. Dishes were incubated in the presence of cytokines for 24 or 48hr.

2.9.2. Transfection of cells growing in suspension

Cells growing in suspension were also transfected by a modified calcium phosphate-DNA precipitate method. Approximately $1x10^7$ cells were collected by centrifugation, the supernatant medium was discarded and the cell pellet resuspended in 1ml of the appropriate $Ca_3(PO_4)_2$-DNA suspension (prepared in the manner described in 2.8.1.) and incubated at room temperature for 1hr. Following this, the volume was increased to 10ml with complete RPMI 1640 medium and the cells introduced into a $25cm^2$ tissue culture flask prior to incubation at 37°C and 5% CO_2 for 4.5-6hr. The cells were then pelleted by centrifugation and the pellets washed with 5ml PBS prior to glycerol shocking as described. After washing twice with PBS, the cells were returned to the incubator in 10ml complete RPMI-1640. After the cells had equilibrated with respect to temperature and CO_2, they were stimulated with the appropriate cytokines for 24 or 48hr.

2.10. Analysis of extracts from transfected cells

2.10.1. Preparation of cytoplasmic extracts from transfected cells

Reporter gene expression was assayed in cytoplasmic extracts from transiently transfected cells. Twenty-four or 48hr after glycerol shock, duplicate monolayers were washed with 5ml ice-cold PBS and the cells collected into 5ml PBS using a cell scraper. Dishes were washed with another 5ml PBS and this was combined with the cell suspension from the first scraping. Transiently transfected suspension cells were collected by centrifugation (1200*g*, 5min) and washed once with 5ml ice-cold PBS. The cells were collected by centrifugation at 1200*g* for 5min at 4°C, supernatants discarded and pellets resuspended in 150μl of ice-cold CAT lysis buffer (250mM Tris-HCl [pH 7.8], 10% [v/v] glycerol, 0.25% [v/v] Nonidet-P40); suspensions were transferred to 1.5ml microcentrifuge tubes and cell membranes were lysed by incubation on ice for 10min. Cell lysis was ensured by three cycles of freeze-thawing (2min dry ice/ethanol bath, 1min 37°C) after which cell debris was removed by centrifugation at 20,000*g* for 12min. Supernatants were transferred to fresh microcentrifuge tubes and stored on ice prior to further analysis.

2.10.2. Protein assay

The Bio-Rad protein assay was employed to assess the protein content of cytoplasmic extracts. Five to ten microlitres of cytoplasmic extract was assayed in 1ml of diluted assay reagent (200μl reagent plus 800μl distilled

water) in a 1ml plastic cuvette. The protein content was estimated by spectrophotometry at 595nm using diluted assay reagent as a blank. From the OD_{595}, the extract concentration could be translated into "units" where 1U of cytoplasmic extract was the volume required to produce an absorbance reading of 1.0 at 595nm.

2.10.3. β-Galactosidase assay

Cells were transiently transfected with β-galactosidase gene expression vectors such that the efficiency of transfection could be assessed. The activity of β-galactosidase in cytoplasmic extracts was assayed by measuring o-nitrophenol production following enzymatic cleavage of the substrate o-nitrophenyl galactoside (ONPG). This reaction was measured colorimetrically since its product is a vivid yellow solution of o-nitophenol.

β-Galactosidase activity was assayed in cytoplasmic extracts prepared as described in 2.10.1.. The assay reaction mixture was prepared in a 1.5ml microcentrifuge tube as follows: 3μl 100 x magnesium buffer (100mM $MgCl_2$, 5M β-mercaptoethanol), 66μl ONPG solution (4mg/ml in sodium phosphate buffer [100ml of 0.1M Na_2HPO_4, adjusted to pH7.3 at 37°C with 0.1M NaH_2PO_4]) and the final volume of 300μl (including cell extract) attained by adding an appropriate volume of sodium phosphate buffer. Reactions were started by adding equal unit amounts (1-5U determined by protein assay) of fresh cytoplasmic extracts and tubes were incubated at 37°C. Samples

were incubated until a yellow colour was evident (30-120min), at this time the reaction was terminated by the addition of 0.5M 1M Na_2CO_3. Incubation at 37°C often resulted in the precipitation of cellular material, this was removed by centrifugation at 20,000*g* for 10min. The supernatants were recovered and the absorbance measured at 495nm. Assay conditions were assessed by the incorporation of 100ng of purified enzyme in the reaction mixture in the place of cytoplasmic extract.

2.10.4. Chloramphenicol acetyl transferase (CAT) assay

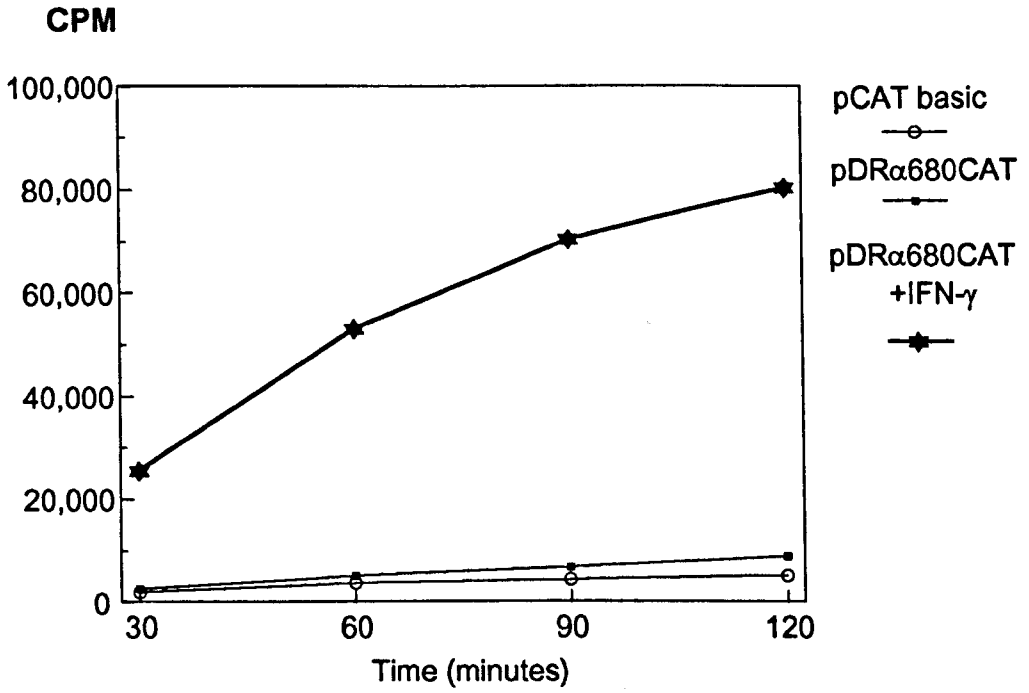
The enzyme assay was performed on fresh cytoplasmic extracts using the direct scintillation diffusion method of Eastman (1987). The number of units of extract was normalised throughout the assay such that 1-5 units (see 2.10.2.) of cytoplasmic extract was incorporated into each reaction mixture and the amount incorporated was equal for each reaction within an assay. Duplicate samples were normalised according to transfection efficiency as determined by the β-galactosidase assay. Since the β-galactosidase promoter was responsive to cytokines (Swingler, PhD thesis (1992)), it was not possible to normalise, with respect to transfection efficiency, between samples which had been subjected to different cytokine treatments.

Extracts were heated at 65°C for 15min prior to their incorporation into the reaction mixture. This was done to inactivate any deacetylases within the samples - bacterial CAT is relatively heat-stable and is unaffected by such treatment. One to five units of extract was mixed in a plastic scintillation vial

with the following reagents: 50μl 5mM chloramphenicol (aq), 25μl 1M Tris-HCl pH7.8, 124μl distilled water and 0.1M Tris-HCl pH 7.8 to give a final volume of 300μl. The substrate, [^{3}H]-acetyl coenzyme A (0.1μCi) was then added to start the reaction and vials were incubated at 37°C after the rapid addition of 4.5ml Econofluor scintillation fluid.

Controls were incorporated to measure i) the passive diffusion of [^{3}H]-acetyl coenzyme A into the solvent phase, ii) the expression of CAT in extracts from cells transfected with the promoterless CAT vector pCATbasic and iii) a positive control was included in which the purified CAT enzyme was employed instead of cytoplasmic extract. As the reaction proceeded, the [^{3}H]-acetylated chloramphenicol reaction product entered the scintillant phase and thus was able to be measured by liquid scintillation counting. Vials were counted for 10s every 30min over a 2hr period. The enzyme reaction was linear over this period of time as shown in **figure 2.8.**

Figure 2.8. Time-course of enzymatic conversion of chloramphenicol to the [^{3}H]-acetate during the chloramphenicol acetyl transferase assay



Notes:

This time-course plot of a typical CAT assay demonstrates the linearity of substrate conversion over the period of the enzyme reaction. This particular assay displays good linear conversion into radioactive product despite the high level of output.

2.11. Analysis of DNA binding proteins

2.11.1. Preparation of nuclear protein extracts

i) Cytokine treatment, harvesting and storage of cells.

Adherent cells were grown to confluence in roller bottles in 100ml complete DMEM which had been pregassed with a 12-15s pulse of CO_2 to yield an atmosphere of approximately 5% CO_2. Suspension cells were also grown in roller bottles in 100ml RPMI-1640 until a density of $2.5x10^6$ cells/ml had been attained. Three or four roller bottles of cells were prepared for each cytokine treatment. Immediately prior to addition of cytokines, the medium on adherent cells was changed and the rollers regassed. Cells were treated with 10^3 U/ml IFN-γ, 200U/ml IFN-α or 10U/ml IFN-γ & 200U/ml IFN-α for 2, 6 or 24hr. Adherent cells were washed with 10ml PBS then harvested by trypsinisation before being stored in 30% (v/v) glycerol in DMEM at -70°C. Suspension cells were harvested by centrifugation, resuspended in 30% (v/v) glycerol in RPMI and stored at -70°C. Frozen cells were stored for upto 6 months prior to preparation of nuclear proteins.

ii) Preparation of nuclear proteins.

Proteins were extracted from nuclei by a method based on that described by Hennighausen and Lubon (1987). All solutions and apparatus were prechilled and homogenisers were autoclaved or baked prior to use.

Frozen preparations of cells were thawed at 37°C then pelleted by centrifugation at 1800rpm (MSE Chilspin) for 10min at 4°C. Pellets (1-1.5ml in volume) were washed with 5ml ice-cold PBS before repelleting then resuspending in 3 pellet volumes of ice-cold buffer A (10mM Hepes-KOH [pH7.9], 10mM KCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.32M sucrose, 0.5mM DTT, 0.5mM PMSF and 2μg/ml each of antipain, leupeptin and pepstatin A). Cells were transferred to a Dounce homogeniser and fully resuspended using 10-12 strokes of a B-pestle. Nonidet-P40 was added to give a final concentration of 0.1% (v/v) and the cell membranes lysed with 4 strokes of the B-pestle. Nuclei were pelleted by centrifugation at 1200*g* for 10min at 4°C and washed twice with three pellet volumes of buffer A (without Nonidet). Lysis was confirmed by visualising nuclei under phase contrast microscopy. The nuclear pellet was resuspended in 0.5-1ml of buffer B (400mM NaCl, 10mM Hepes-KOH [pH 7.9], 1.5mM $MgCl_2$, 0.1mM EGTA, 5% [v/v] glycerol, 0.5mM DTT, 0.5mM PMSF) and transferred to a sterile glass bijou. The suspension was stirred gently at 4°C for 30min to elute the proteins from the nuclei. Nuclear debris was removed by centrifugation at 100,000*g* for 1hr. Supernatants were dialysed against 100 volumes of buffer C (20mM Hepes-KOH [pH 7.9], 75mM NaCl, 0.1mM EDTA, 20% [v/v] glycerol, 0.5mM DTT, 0.5mM PMSF) for 1.5-3hr at 4°C. Precipitated material was removed by centrifugation (25,000*g* for 15min) and the supernatants assayed for protein concentration by the Bio-Rad protein assay (see section 2.9.2.). Nuclear proteins were aliquoted in volumes of 20-500μg and stored for up to six months at -70°C until use.

2.11.2. Preparation of radio-labelled DNA probes

DNA probes for use in bandshift and footprinting assays were prepared from either double-stranded oligonucleotides or from larger fragments of DNA excised from plasmid vectors. Techniques were employed such that the DNA was radiolabelled at one end to high specific activity using γ- or α-[^{32}P]-nucleoside triphosphates. Polynucleotide kinase from T4 bacteriophage was used with [γ-^{32}P]-ATP to phosphorylate 5'-hydroxyl groups whereas the 3' end was labelled by end-filling with reverse transcriptase and one or two [α-^{32}P]-dNTPs. A schedule for the radiolabelling of all probes used in bandshift and footprinting analysis is given in **figure 2.9**.

i) Preparation of plasmid DNA for labelling.

The promoter sequences studied were cloned into the plasmid vector pBluescript II KS^{+}. Restriction endonucleases were chosen such that a fragment of the promoter of convenient size could be excised from the vector and radiolabelled at a site which was a suitable distance from the DNA binding protein consensus sequences within the promoter. In order that the DNA was radiolabelled at one end, the two restriction enzymes selected were different.

DNA was prepared according to the method described by Goodwin (1990). Fifty micrograms of the plasmid DNA was digested with the first restriction enzyme in 100μl of buffer (see 2.8.4.[i]). Twenty units of CIAP was then added and the mixture incubated at 37°C for 30min before the reaction was terminated by the addition of 10μl 100mM EDTA and 10μl 10% (w/v) SDS.

The DNA was extracted twice with phenol/chloroform/isoamyl alcohol then ethanol precipitated. After washing and air-drying, the pellet was resuspended in 10µl TE or sterile distilled water (containing 50µg/ml RNase A) and stored at -20°C.

ii) T4 Polynucleotide kinase reaction.

Two microlitres of cut, dephosphorylated plasmid DNA or annealed oligonucleotide (20pmol) were incubated at 37°C for 45min with 10µl [γ-^{32}P]-ATP (100µCi; 5000Ci/mmol) and 1µl (10U) T4 PNK in a 50µl reaction volume containing 50mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA. The reaction was terminated by the addition of EDTA and SDS and the mixture phenol/chloroform extracted and ethanol precipitated. The pellet was resuspended 20µl TE or water and 3M sodium acetate added to give a final concentration of 0.3M. The DNA was then reprecipitated with 100% ethanol, washed twice with 70% ethanol, air-dried and resuspended in 10µl TE before digesting with the second restriction enzyme in a final buffer volume of 50µl. The reaction was terminated by the method described, 12µl of formamide load dye (80% [v/v] deionised formamide, 10mM NaOH, 1mM EDTA, 0.1% [w/v] xylene cyanol, 0.1% [w/v] bromophenol blue) was added and the tube vortexed. The labelled DNA fragment was separated from the vector by polyacrylamide gel electrophoresis through a 5% (50-500bp) or 10% (20-30bp) gel in 1xTBE (see section 2.8.3.[iii]). After separation, the labelled DNA fragments were identified by autoradiography and the desired fragment recovered from the polyacrylamide gel by the "crush and soak"

method (section 2.8.3.[iv]). The addition of 5μg of carrier tRNA enabled the efficient precipitation of oligonucleotide probes. DNA was precipitated with 100% ethanol, washed with 70% ethanol and air-dried as described. Probes were resuspended in 200μl TE or water to give a specific activity of 50,000-150,000cpm/μl ($3.5x10^7$-$1x10^8$cpm/μg) and stored at -20°C for upto 10 days.

iii) End-filling with reverse transcriptase.

Two microlitres (≈10μg) of cut, dephosphorylated plasmid DNA was incubated at 37°C for 1hr with 100μCi (10μl) [α-^{32}P]-dCTP, 100μCi (10μl) [α-^{32}P]-dATP (each at 6000Ci/mmol) and 40U (2μl) AMV reverse transcriptase in 50μl of buffer containing 10mM Tris-HCl (pH 8.3), 80mM KCl, 10mM $MgCl_2$ and 12mM β-mercaptoethanol. The reaction mixture was then purified, digested with the second restriction enzyme and separated from the vector by electrophoresis in the same manner as that described in section 2.11.2.(ii) (above). DNA was eluted from the polyacrylamide gel into 2.5ml low salt (LS) buffer (0.2M NaCl, 20mM Tris-HCl [pH 7.6], 1mM EDTA) by shaking overnight at 37°C. Centrifugation at 2000*g* for 10min was performed such that the solid acrylamide could be removed. The supernatant was then collected; a further 1ml LS buffer added to the acrylamide and the tube was vortexed before centrifuging again. The supernatants were combined and pipetted onto an equilibrated Schleicher and Schuell Elutip D column. After washing the column with 5ml LS buffer, the radiolabelled DNA was eluted into 0.4ml high salt (HS) buffer (1.0M LiCl, 20mM Tris-HCl [pH7.6], 1mM EDTA). The DNA was

precipitated with 100% ethanol at -70°C for 1hr, collected by centrifugation and washed twice with 70% ethanol prior to air-drying and resuspending in 200µl TE or sterile distilled water to give a specific activity of 70,000-150,000cpm/µl (2.3 to 4.9x10^{7}cpm/µg). Probes were stored at -20°C for upto 10 days before use.

2.11.3. Bandshift assay

In this type of experiment (also referred to as gel retardation, electromobility shift assay etc) nuclear proteins were incubated with radiolabelled probes corresponding various parts of the promoter of interest or a larger promoter fragment probe. During polyacrylamide gel electrophoresis, the labelled probe to which protein, ie transcription factors, is bound has its progress through the gel hindered by the presence of bound proteins and so does not travel as far as unbound probe. The binding of transcription factors to the labelled probe is observed after autoradiography as a novel band/s above the free probe.

The specificity of the binding of such complexes to the probe could be assessed by the incorporation of excess unlabelled (cold) DNA of exactly the same sequence as the probe into the binding reaction mixture. Such sequestration of complexes by the unlabelled competitor DNA results in the disappearance of bands or reduction in band intensity upon autoradiography.

Use and variation of this technique facilitated the study of ubiquitous and

cytokine-inducible DNA binding factors in the cell lines under investigation with a view to assessing whether differences in HLA-DR expression observed at the cell surface could be reflected at the level of protein-DNA interactions.

Crude nuclear extracts (5-20μg) were preincubated for 20min at room temperature with 1-8μg poly (dI-dC):poly (dI-dC) (from 4μg/μl stock dissolved in poly dI-dC buffer [50mM NaCl, 10mM Tris-HCl (pH 7.6), 1mM EDTA]) and 1μg pUC13 plasmid DNA in "gel retardation buffer" (20mM Hepes-NaOH [pH7.6], 4% Ficoll, 5mM $MgCl_2$, 40mM NaCl, 0.1mM EDTA, 0.5mM DTT) in a final volume (including probe) of 20μl. One microlitre (20,000cpm) of probe solution was then added and the mixture incubated for a further 45-60min on ice. Samples were then loaded into the wells of a pre-run (150V, 20min) 5% (w/v) polyacrylamide gel containing 0.2xTBE and electrophoresed at 150V for 3-6hr depending on the size (in base pairs: 2-3hr for oligonucleotides; 4-6hr for DNA fragments) of the probe employed (see section 2.8.3[iii]). Formamide load dye was also electrophoresed in order to assess the progress of separation. After completion of electrophoresis, gels were soaked in 10% (v/v) glycerol for 30min prior to drying under vacuum at 80° C for 1hr. Dried gels were autradiographed against Fuji RX 100 X-ray film at -70°C for 12hr to 3days. After developing and fixing the film, dark bands corresponding to free probe and probe to which protein complexes were bound could be visualised. The specificity of the binding of protein complexes to radiolabelled probes was determined by performing competition bandshift assays. This variety of experiment was performed in the same manner as the standard assay except "competitor" DNA

in varying molar excesses to the amount of probe DNA was incorporated into the pre-incubation mixture. Competitor DNA was defined as an unlabelled piece of DNA whose sequence was the same as the probe or a piece of unlabelled DNA whose sequence was related to that of the probe's.

Competition of binding was defined as the disappearance of a band or the significant reduction in intensity of a band upon incorporation of competitor DNA in the incubation mixture.

2.11.4. Maxam and Gilbert sequencing of fragment probes

End-labelled fragment probes labelled on one strand were sequenced chemically to provide a reference for footprinting reactions. DNA for sequencing was prepared in the following manner: a volume of end-labelled probe sufficient to yield 60,000cpm defined by Cérènkov counting, was increased to 20μl with sterile distilled water and 2μl 3M sodium acetate added. The DNA was precipitated with an equal volume of isopropanol prior to washing with 70% ethanol and drying briefly under vacuum. Pellets were resuspended in 40μl sterile distilled water and the probe distributed between the four chemical cleavage reactions as shown in table 2.5. The chemical cleavage reactions were performed as described in table 2.5. Following the final evaporation step, the cleaved DNA was resuspended in 5μl formamide load dye and stored at -20°C prior to electrophoresis through sequencing gels. The compositions of solutions employed throughout the sequencing procedure are given below.

Composition of Maxam & Gilbert sequencing buffers:

DMS reaction buffer

50mM sodium cacodylate, pH 8.0

1M β-mercaptoethanol

100μg/ml tRNA

DMS stop buffer

1.5M sodium acetate

1mM EDTA, pH 7.0

100μg/ml tRNA (after filter-sterilising)

Hydrazine stop buffer

0.3M sodium acetate, pH 7.0

0.1mM EDTA

25μg/ml tRNA (after filter sterilising)

Table 2.5. Outline of Maxam and Gilbert chemical sequencing reactions

Component	Specificity of DNA cleavage G	G+A	T+C	C
DNA, µl	5[a]	10[b]	10[b]	5[a]
DMS reaction buffer, µl	200	—	—	—
Water, µl	5	—	10	10
5M NaCl, µl	—	—	—	5
Base-specific modification reactions				
DMS, µl	1	—	—	—
Formic acid, µl	—	25	—	—
Hydrazine, µl	—	—	30	30
Reaction time (min)	4	10	15	8
Reaction stopped rapidly by dilution with stop buffer and -20°C ethanol and immersing in dry ice/ethanol bath for 10min				
DMS stop buffer, µl	50	—	—	—
Hydrazine stop buffer, µl	—	200	200	200
100% ethanol, µl	750	750	750	750
DNA collected by centrifugation at 20,000*g* for 15min, washed twice with 1ml 70% ethanol and air-dried.				
Remaining reagents removed by re-precipitation (as above)				
Water,µl	200	200	200	200
3M sodium acetate, µl	20	20	20	20
100% ethanol, µl	500	500	500	500
Strand scission reaction at modified bases by incubation at 90°C for 30min in 10% (v/v) piperidine				
10% (v/v) piperidine, µl	70	70	70	70
70 µl water added after incubation, vortexed and evaporated.				
All traces of piperidine removed by evaporation twice from water				
sterile water, µl	60	60	60	60
sterile water, µl	50	50	50	50

[a]10,000, [b]20,000 Cérènkov cpm

2.11.5. DNase I footprinting

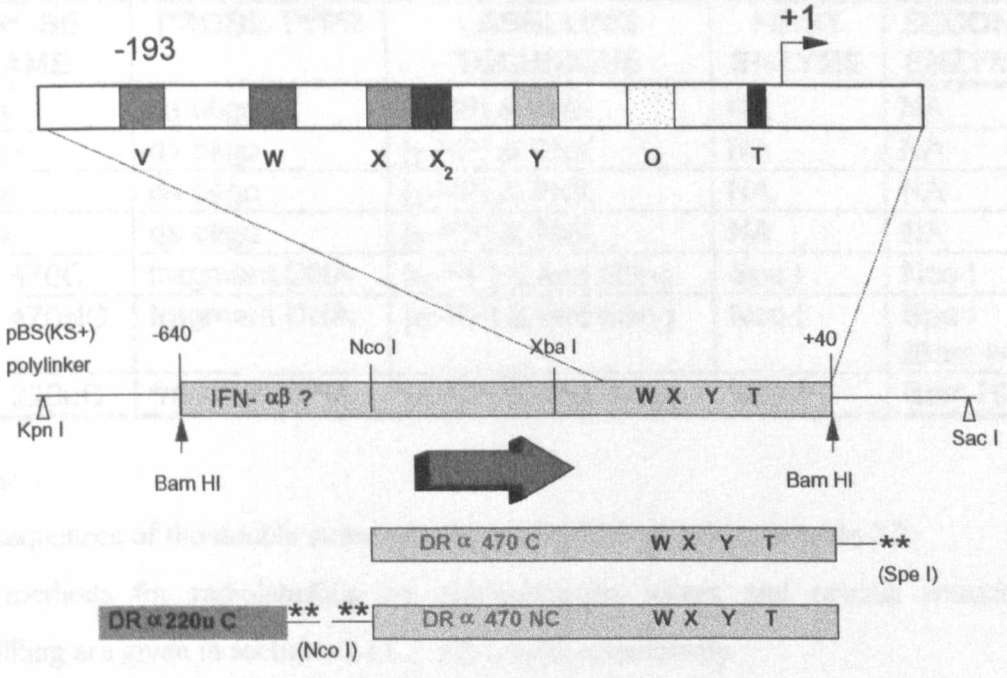
The method employed was based on that described by Goodwin (1990). Probes employed were fragments of DNA derived from the HLA-DRα promoter. End-labelled probes were partially digested with DNase I (bovine pancreatic) in the presence of bound nuclear proteins. Crude nuclear extract (0-200μg) was incubated with 4μg poly (dI-dC):poly (dI-dC) and 2-5*f*mol of end-labelled DNA fragment ($2x10^4$cpm) in 100μl buffer containing 20mM Hepes-KOH, pH 7.6, 40mM NaCl, 2mM $CaCl_2$, 5mM $MgCl_2$, 1mM DTT, 0.5mM $ZnCl_2$, 10% (v/v) glycerol. This was incubated on ice for 60min then digested with 1U DNase I for 1min at room temperature. The reaction was terminated by the rapid addition of 100μl DNase stop buffer (2% [v/v] SDS, 10mM EDTA [pH 8.0], 0.1mg/ml tRNA) followed by 200μl phenol/chloroform/isoamyl alcohol (25:24:1) and mixed by vortexing. The phases were separated by centrifugation at 20,000*g* for 10min and the upper phase collected. The solvent phase was back-extracted with 100μl stop buffer followed by vortexing and centrifugation. The aqueous phases were pooled and re-extracted with 300μl phenol/chloroform/isoamyl alcohol as above. The fragmented DNA was ethanol precipitated then collected by centrifugation at 20,000*g* for 15min. DNA pellets were washed twice with 70% ethanol and dried briefly under vacuum prior to resuspending in 5μl formamide load dye. The DNA was then denatured by heating at 90° C for 10min followed by snap-freezing in dry ice/ethanol. Samples were thawed on ice before being loaded into the wells of an 8% sequencing gel which had been pre-electrophoresed at 1500V (85W, 50mA).

2.11.6. Preparation of sequencing gels

DNase I digested DNA form footprinting reactions and DNA cleaved by the Maxam and Gilbert sequencing method was resolved by electrophoresis through 8% (w/v) acrylamide (19:1 acrylamide:bis-acrylamide) 8M urea gels.

Gels containing 1xTBE were prepared, filtered, then poured between 31.0x38.5cm glass plates separated by 0.4-1.2mm wedged spacers. Polymerisation was catalysed by the addition of 850µl 10% (w/v) ammonium persulphate and 85µl TEMED (per 150ml liquid gel mixture) prior to pouring the gel. Gels were electrophoresed in a Gibco-BRL model S2 sequencing tank at 85W (approximately 1500V, 80mA) in 1xTBE for 40-60min to pre-run and warm the gel. The samples from footprinting and sequencing were heated to 95°C for 3min then snap-frozen in a dry ice/ethanol bath prior to thawing on ice and loading into the wells of the pre-run gel. Samples were electrophoresed at the same settings for 4-5hr until the required degree of separation was achieved. Following electrophoresis, one glass plate was removed and the gel transferred to a sheet of double-thickness 3M paper. The gel was covered with cellophane and dried under vacuum at 80° C for 3hr. Dried gels were autoradiographed against Fuji RX film with an intensifying screen for 3-7days at -70°C.

Figure 2.9. Strategy for labelling fragment probes used in bandshift and footprinting analyses



Notes:

Fragment probes were prepared from the 680bp fragment of the HLA-DRα promoter cloned into the Bam HI site of the polylinker of the phagemid pBluescript (II) KS^+. Fragments were generated by restriction endonuclease digestion according to the method of Goodwin (1990) [section 2.11.2. (i) & (ii)].

The restriction enzymes employed to generate the sites for end-filling are shown.

** indicates the incorporation of two [α-^{32}P]-labelled nucleoside phosphates by reverse transcriptase-catalysed end-filling (section 2.11.2 [iii]).


indicates the direction of transcription from the start site.


indicates the start of transcription.

The lengths of probes and the strand labelled are indicated by number and C or NC (for coding and non-coding, respectively). The name DRα-uC indicates that this probe covers the upstream extreme of the promoter region.

Table 2.6. Radioactive labelling of oligonucleotides and DNA fragments employed in bandshift and footprinting analyses

PROBE NAME	PROBE TYPE	LABELLING TECHNIQUE	FIRST ENZYME	SECOND ENZYME
V box	ds oligo	[γ-^{32}P] & PNK	NA	NA
W box	ds oligo	[γ-^{32}P] & PNK	NA	NA
X box	ds oligo	[γ-^{32}P] & PNK	NA	NA
Y box	ds oligo	[γ-^{32}P] & PNK	NA	NA
DRα 470C	fragment DNA	[α-^{32}P] & end filling	Spe I	Nco I
DRα 470NC	fragment DNA	[α-^{32}P] & end filling	Nco I	Spe I /Bam HI
DRα 220uC	fragment DNA	[α-^{32}P] & end filling	Nco I	Bam HI

Notes:

The sequences of the double stranded oligonucleotides are given in table 2.2.

The methods for radiolabelling by polynucleotide kinase and reverse transcriptase end-filling are given in sections 2.11.2. (ii) & (iii), respectively.

NA=not applicable.

CHAPTER 3

CHAPTER 3: ANALYSIS OF NUCLEAR DNA BINDING PROTEINS INVOLVED IN THE INTERFERON-γ-INDUCED EXPRESSION OF HLA-DRα

3.1. Introduction

The nature of transcription factors which bound to the HLA-DRα promoter was investigated. Since the expression of this gene is tightly controlled by cytokines and in a tissue-specific manner, cell lines were treated with a variety of cytokines prior to their being harvested and nuclear proteins being extracted by the described method.

In non-haematopoietic cells, surface expression of HLA-DR and other class II MHC genes can only be observed after exposure to interferon-γ. The human tumour cell lines under investigation here responded variably to IFN-γ treatment in this respect: using flow cytometric analyses, the cell lines colo 201 colo 205 and U373MG showed good induction of class II MHC after IFN-γ treatment, whereas the cell lines caco 2 and LS180 could not be induced to express HLA-DR under the same conditions (Darley, *et al* (1993)). It was this difference in ability for IFN-γ-induced class II expression which was the prime target for investigation here. Experimentation followed the hypothesis that those cell lines which were unable to be induced for class II MHC expression were lacking in components of the transcription factors required for activation of expression.

The first part of the study was to assess the populations of HLA-DRα

promoter binding proteins before and after IFN-γ-treatment of the cell lines employed. This was achieved by performing bandshift assays with radiolabelled probes which corresponded to the W and X boxes of the HLA-DRα. These elements were chosen due to their reported importance in the IFN-γ-induced expression of class II MHC. The W box had been implicated in IFN-γ-induced transcription when its mutation in reporter gene assays reduced expression levels to basal (Basta, *et al* 1988). Also, the X box had been shown to bind IFNEX in response to IFN-γ-treatment of cells. Although absolutely necessary for the transcription of HLA-DRα, the Y box's involvement was considered more as a general one (e.g. for the binding of CAAT transcription factor) and as such it was not investigated in this respect.

3.1.1. Cell lines

The cell lines employed initially in this study were those which were derived from human tumours as described in section **2.1**.; these being colo 201, colo 205, U373MG, LS180 and caco 2. None of these cell lines displayed constitutive surface expression of class II MHC and each showed a distinct response to IFN-γ-treatment as regards surface expression of class II MHC antigens (see **table 2.1**). The objective of these initial experiments was to assess whether the differences in surface expression of HLA-DR was reflected in differences in the populations of specific transcription factors which bind to discrete regions of the HLA-DRα promoter. To facilitate this, double stranded oligonucleotide probes corresponding to the individual *cis*-acting elements of

the promoter were employed as radiolabelled probes (prepared by T4 polynucleotide kinase labelling) in bandshift assays. Crude nuclear extracts from the cell lines employed were prepared before or after IFN-γ-stimulation by the method described. The bandshift assay was performed in each case by the method of Goodwin (1990) described in section **2.11.3.**

3.2. Analysis of nuclear proteins binding to the HLA-DRα W (Z) box

The individual *cis*-acting sequences which are conserved within all class II MHC promoters, ie W, X and Y boxes, have been studied extensively since they were identified by sequence homology. Their cooperation in activation of constitutive and cytokine induced transcription of HLA-DRα and their individual rôles in promoter activity have been somewhat elucidated. Deletion or mutation of just one of the elements abolishes the ability of the HLA-DRα to direct constitutive and IFN-γ-induced transcription. One study (Basta, *et al.*, 1988) employing reporter gene analysis showed that deletion or mutation of just the W (Z) box was sufficient to reduce transcriptional activity of the promoter to its basal level. Because of this, initial bandshift studies focussed on the binding of proteins from nuclear extracts to the W box.

A double stranded DNA oligonuceotide whose sequence incorporated the HLA-DRα W box consensus was synthesised. The sequence of this oligonucleotide is as follows:

5'-CGTGTCCT**GGACCC**TTTGCAAGAACCG-3'
3'-GCACAGGA**CCTGGG**AAACGTTCTTGGC-5'

The part of the oligonucleotide shown in **bold type** indicates the consensus W box element which is considered to be the transcription factor binding sequence.

This was radiolabelled using T4 bacteriophage polynucleotide kinase and employed in bandshift experiments using nuclear protein extracts from IFN-γ-treated and untreated tumour cell lines. Assays were performed at least in duplicate with nuclear protein extracts from three independent cell preparations. The cells were grown to high density and treated with 1000U/ml IFN-γ for 6 or 24hr prior to harvesting and extraction of nuclear proteins. This concentration of IFN-γ was used to induce the maximal response. The time points chosen were intended to "capture" any changes in the populations of DNA binding proteins and were selected from published observations of HLA-DRα mRNA levels and surface expression (refer to section **1.6.**).

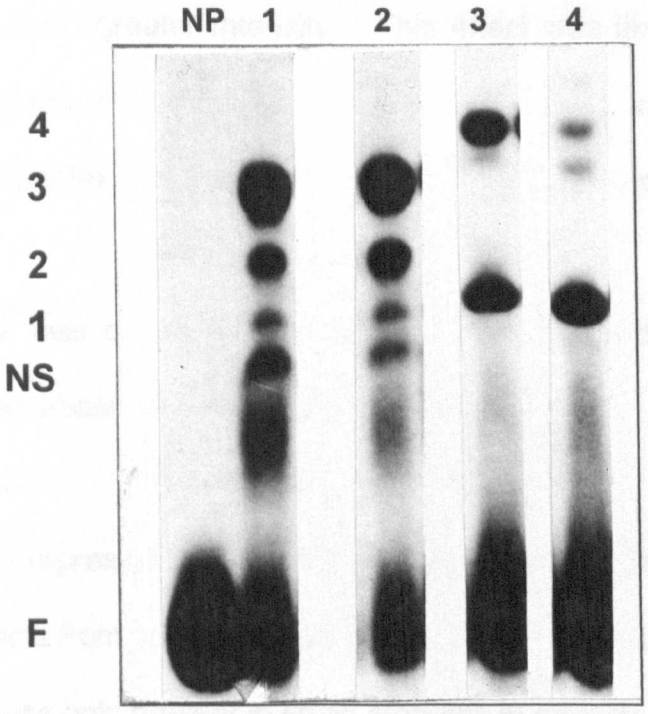
3.2.1. Constitutive binding of protein complexes to the HLA-DRα W-box probe

Colo 201, colo 205, LS180 and caco 2

In a series of experiments, nuclear extracts were used from the cell lines colo 201, colo 205, LS180 and caco 2. The constitutive binding of nuclear factors to the HLA-DRα W box probe in shown in **figure 3.1.**

The free probe is visualised as the dense band at the bottom of the autoradiograph, ie, the fastest migrating band. The next fastest migrating band (labelled NS) is a non-specific protein-DNA complex, this being confirmed in competition bandshift experiments (see section **3.4.**).

Figure 3.1. Constitutive binding of nuclear factors to the HLA-DRα W box probe



Bandshift experiment employing nuclear extracts from unstimulated colorectal tumour cell lines colo 201, colo 205, LS180 and caco 2 (lanes 1 to 4, repsectively) and a radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα W box. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). The free probe is indicated by "F"; NS=non-specific complex; NP=no protein control. Those binding complexes shown to be specifically bound are numbered according to their respective mobilities.

Band 1 was the first of the specific protein-DNA complexes. Its presence was ubiquitous amongst all cell lines investigated and was independent of cytokine treatment of cells. In colo 201 and colo 205 nuclear extracts this complex appeared to be less evident than in the LS180 and caco 2 where the band was of much greater intensity.. This effect was likely to be due to this complex being the most abundant in uninduced caco 2 and LS180 whereas it was a minor complex in nuclear extracts of colo 201 and colo 205.

Band 2 was observed in colo 201, colo 205 and, to a lesser extent, LS180, yet was absent from caco 2 extracts.

Band 3 represented the most abundant complex binding to the W box probe in extracts from untreated colo 201 and colo 205 yet was absent in LS180 extracts and was only present in small amounts in extracts from caco 2.

Uninduced LS180 and caco 2 extracts also contained a low mobility complex (band 4) which was absent in uninduced colo 201 and colo 205. This complex was of equal intensity to band 1 in LS180 and it was assumed that it was of equal abundance in this cell line. In contrast, it was of less intensity than band 1 in caco 2 and was, therefore, less copious.

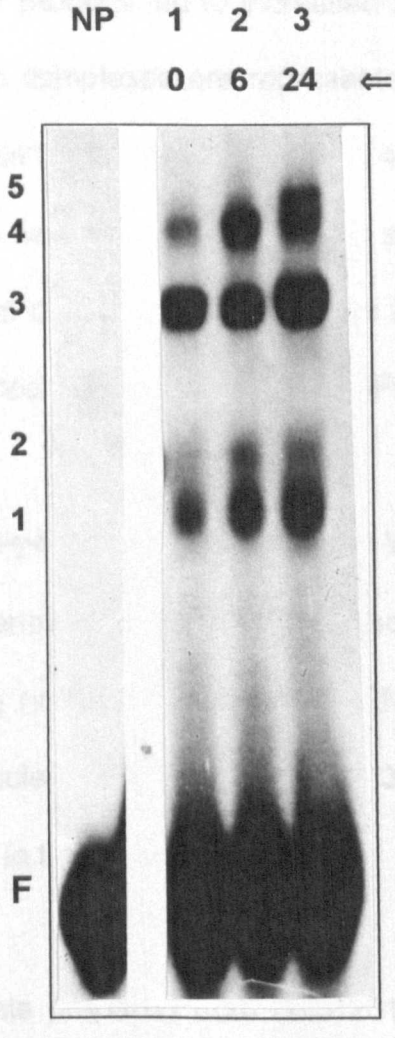
U373MG

Nuclear extracts from U373MG were not assessed in the same assays as the colorectal tumour cell lines and the results cannot be compared. Bandshift experiments with the same W box oligonucleotide probe revealed the association of four complexes from uninduced cells (see **figure 3.2**). This figure also shows the formation of binding complexes observed with nuclear proteins from IFN-γ-stimulated U373MG.

Constitutive binding of U373MG nuclear factors:

Unstimulated U373MG contained four factors which bound to the W box probe, these are labelled as bands 1, 3 and 4 in figure 3.2. Of these complexes, the band which is represented as "3" in the figure was observed as a doublet. The proteins which caused the formation of these complexes and that/those whose binding resulted in band 1 were observed as the being equally abundant.

Figure 3.2. Binding of nuclear factors from unstimulated and IFN-γ-stimulated U373MG to the HLA-DRα W box probe



Bandshift experiment employing nuclear extracts from unstimulated and IFN-γ-stimulated U373MG astrocytoma cell line; cells were treated for 0, 6 or 24 hr with 1000U/ml IFN-γ (lanes 1 to 3, respectively). A radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα W box was employed. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). The free probe is indicated by "F"; NP=no protein control. Those binding complexes shown to be specifically bound are numbered according to their respective mobilities.

IFN-γ-induced U373MG factor binding

The treatment of U373MG with 1000U/ml IFN-γ for 6 and 24hr prior to preparation of nuclear proteins led to increased association of factors with this probe. Novel binding complexes are represented as bands 2 and 5 in figure 3.2. The binding of the factor resulting in band 4 was increased after 6hr IFN-γ treatment and band 5 was observed after 24 hr stimulation as a complex which migrated with retarded mobility to band 4. The association of factors resulting in band 3 also appeared to increase after 24hr IFN-γ stimulation of cells.

3.2.2. Binding of IFN-γ-induced factors to the W box

In the same series of experiments as discussed above, bandshifts were performed employing nuclear extracts from IFN-γ-treated cells alongside the unstimulated cell nuclear extracts. **Figure 3.3.** shows the result of one bandshift experiment in this series.

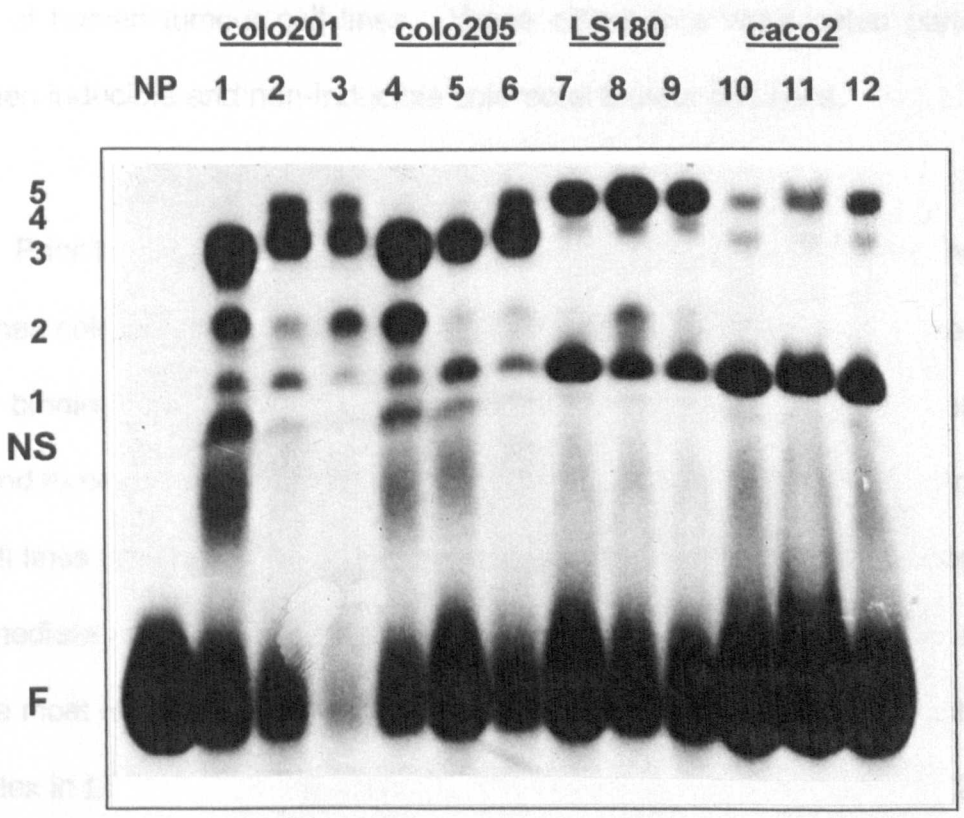
Nuclear extracts prepared from colo 201 and colo 205 and contained novel W box binding factors upon stimulation with IFN-γ. Colo 201 treated with IFN-γ for 6 or 24hr and colo 205 treated for 24hr possessed a factor which migrated more slowly than the most retarded complex in the uninduced cells (figure 3.3., band 4). This complex migrated with marginally greater mobility than the least mobile complexes of LS180 and caco 2 (band 5).

The appearance of this complex in stimulated colo 201 and colo 205

coincided with the diminution in intensity of band 2 compared to the untreated. In colo 205, this complex disappeared after treatment of the cells with IFN-γ, yet it was observed to reassociate after 24hr stimulation of colo 201. It was postulated that the complex resulting in the appearance of band 4 was a derivative of that complex which formed band 2.

No differences in the binding of nuclear factors from LS180 and caco 2 were observed after treatment of these cells with IFN-γ

Fig. 3.3. Binding of nuclear factors to the HLA-DRα W box probe after treatment of cells with IFN-γ



Bandshift experiment employing nuclear extracts from unstimulated and IFN-γ-stimulated colorectal tumour cell lines colo 201, colo 205, LS180 and caco 2 (as indicated above lane markers) and a radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα W box. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). Lanes 1, 4, 7 and 10: extracts from unstimulated cells. Lanes 2, 5, 8, and 11: cells treated with 1000U/ml IFN-γ for 6hr. Lanes 3, 6, 9 and 12: cells treated with 1000U/ml IFN-γ for 24hr. The free probe is indicated by "F"; NS=non-specific complex; NP=no protein control. Those binding complexes shown to be specifically bound are numbered according to their respective mobilities.

3.2.3. Summary of results

The binding of nuclear proteins to a probe corresponding to the HLA-DRα W revealed differences in the transcription factor population of a panel of human tumour cell lines. These differences were noted particularly between inducible and non-inducible colorectal tumour cell lines.

Prior to IFN-γ-stimulation of the colorectal tumour cell lines, the inducible cell lines colo 201 and colo 205 contained nuclear proteins which formed three major binding complexes. The uniducible lines, LS180 and caco 2 contained two and three binding complexes, respectively and of these one was common to all cell lines (the fastest migrating specific complex termed band 1), whereas an intermediate migratory complex in caco 2 appeared to have the same mobility as the most retarded band in colo 201/205 extracts (band 3). The least mobile complex in LS180 and caco 2 was absent in nuclear extracts from colo 201 and colo 205.

It was not possible to compare the W box binding complexes form uniduced U373MG with these colorectal tumour cell lines, in this situation the W box probe bound four nuclear factors (bands 1, 3 and 4 in figure 3.2).

After stimulation of cells with IFN-γ, no differences in the binding of factors was observed with LS180 and caco 2. However, colo 201 and colo 205 both exhibited the binding of a novel complex (band 4 in figure 3.3.), migrating

with slower mobility than those observed prior to stimulation. Its appearance was noted after 6hr IFN-γ treatment of colo 201 and 24hr stimulation of colo 205. This complex migrated with marginally greater mobility than the slowest migrating band observed with unstimulated and IFN-γ-treated LS180 and caco 2. The complex which gave rise to band 2 in unstimulated colo 201 and colo 205 was greatly diminished upon IFN-γ treatment although its reappearance was noted after 24hr IFN-γ stimulation of colo 201.

A novel retarded complex from U373MG was also noted to bind to the W box probe after 24hr IFN-γ stimulation of this cell line. In contrast with the inducible colorectal tumour cell lines, this appearance was not coincident with the reduced binding of any complex observed prior to IFN-γ stimulation.

3.3. Analysis of nuclear proteins binding to the HLA-DRα X box

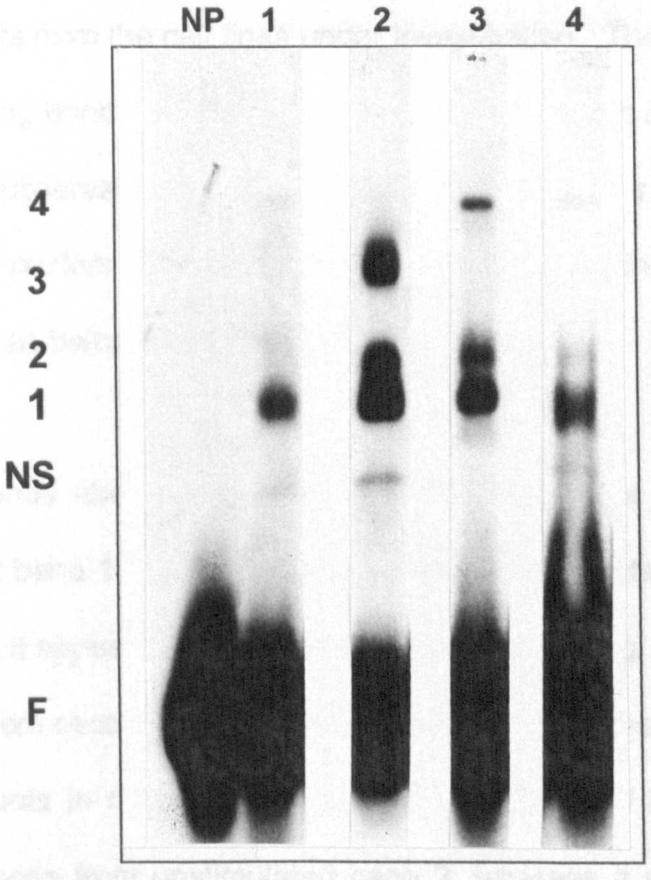
The study into X box binding factors was undertaken in order to determine whether any novel factors were observed upon IFN-γ treatment of the colorectal tumour cell lines under investigation here. The experimentation was performed in the same manner as that described for the W box study. The sequence of the double stranded oligonucleotide probe employed in bandshift experiments is given below:

5'-CC**CCTAGCAACAGATGCGTCATC**TC-3'
3'-GG**GGATCGTTGTCTACGCAGTAG**AG-5'
<------X------><-X_2->

The consensus X box and adjoining X_2 box are indicated by **bold print.**

Probes were radiolabelled using [γ-^{32}P] ATP and polynucleotide kinase as described. Cells were grown and treated with IFN-γ for 6 or 24hr or untreated prior to harvesting and extraction of nuclear proteins. **Figure 3.4.** shows the result of one bandshift experiment in which nuclear extracts from untreated cells were incubated with the labelled X box probe.

Fig. 3.4. Interaction of DNA binding proteins from unstimulated colorectal tumour cell lines with the HLA-DRα X box probe



Bandshift experiment employing nuclear extracts from unstimulated colorectal tumour cell lines colo 201, colo 205, LS180 and caco 2 (lanes 1 to 4, respectively) and a radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα X box. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). The free probe is indicated by "F"; NS=non-specific complex; NP=no protein control. Those binding complexes shown to be specifically bound are numbered according to their respective mobilities.

3.3.1. Constitutive binding of proteins to the HLA-DRα X box

Colo 201, colo 205, LS180 and caco 2

Constitutive binding was represented by that which is observed from the untreated cells (see **figure 3.4.**) A number of complexes were observed in the nuclear extracts from the cell lines under investigation. The free probe was the fastest migrating band at the bottom of the autoradiograph and was the densest of the bands observed - confirming that it was present in each incubation in excess of the nuclear proteins. The next fastest migrating band (termed NS) was identified as being due to non-specific binding.

The bands above were specifically bound to the probe. The first of these, termed band 1, was present in the nuclear extracts of all the untreated cells although it appeared as a marginally faster migrating, more diffuse band in the extracts from caco 2. The next most retarded band (band 2) was present in copious amounts in nuclear extracts form colo 205 and LS180 and in lesser amounts in those from unstimulated caco 2, whereas it was absent from the nuclear extracts of colo 201.

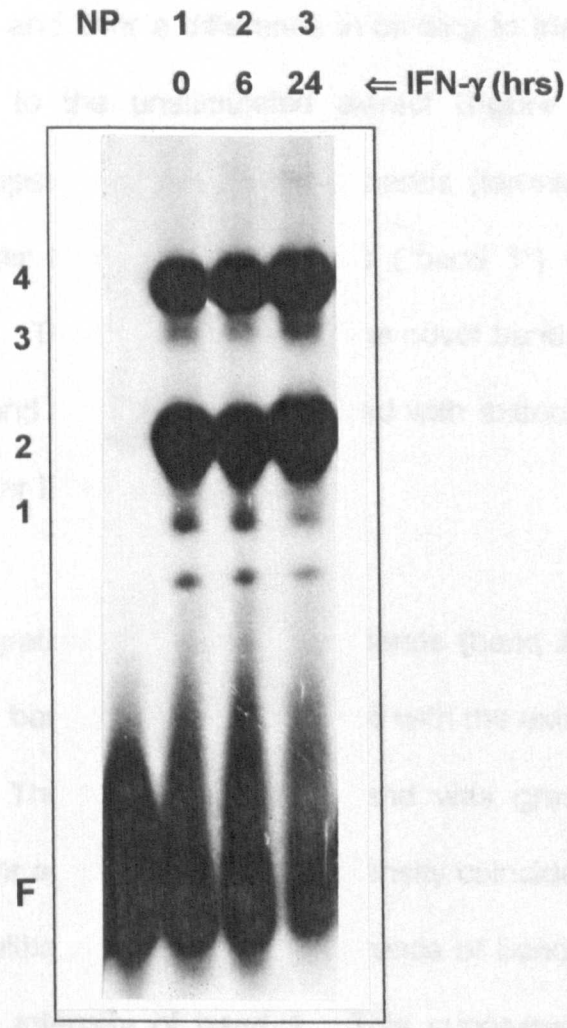
In the extracts from unstimulated colo 205 there was a very intense band (band 3) which was unique to this cell line under these conditions. There was a faint band (band 4) which was present solely in extracts from untreated LS180.

U373MG

As was the case with the study into the W box, nuclear extracts from U373MG were not assayed in the same experiments as those from colorectal tumour cell lines. Bandshifts with the X box revealed the association of four nuclear protein complexes (shown in **figure 3.5.**). Comparisons could not be made between these factors and those observed from the colorectal cell lines. Of the four complexes observed binding to this probe, two (bands 2 and 4) were the most abundant. The appearance of bands 1 and 3 was very faint, presumably due to their relatively low concentrations in this population of X box binding proteins.

There was no difference in the association of factors to the X box probe after treatment of U373MG with IFN-γ (figure 3.5).

Figure 3.5. Binding of U373MG nuclear proteins to the HLA-DRα X box probe



Bandshift experiment employing nuclear extracts from unstimulated and IFN-γ-stimulated U373MG astrocytoma cell line; cells were treated for 0, 6 or 24 hr with 1000U/ml IFN-γ (lanes 1 to 3, respectively). A radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα X box was employed. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). The free probe is indicated by "F". Those binding complexes shown to be specifically bound are numbered according to their respective mobilities.

3.3.2. Binding of factors to the HLA-DRα X box probe after stimulation of cells with IFN-γ

In nuclear extracts from colo 201 which had been treated which 1000U/ml IFN-γ for 6 and 24hr a difference in binding to the X box probe was observed compared to the unstimulated extract (**figure 3.6.**). This was manifested in the appearance in two novel bands (termed A and B) which migrated much slower than the single band ("band 1") observed with the unstimulated extracts. The most retarded of the novel bands migrated with the same mobility as "band 5" which was observed with extracts from LS180 and caco 2 before and after IFN-γ stimulation.

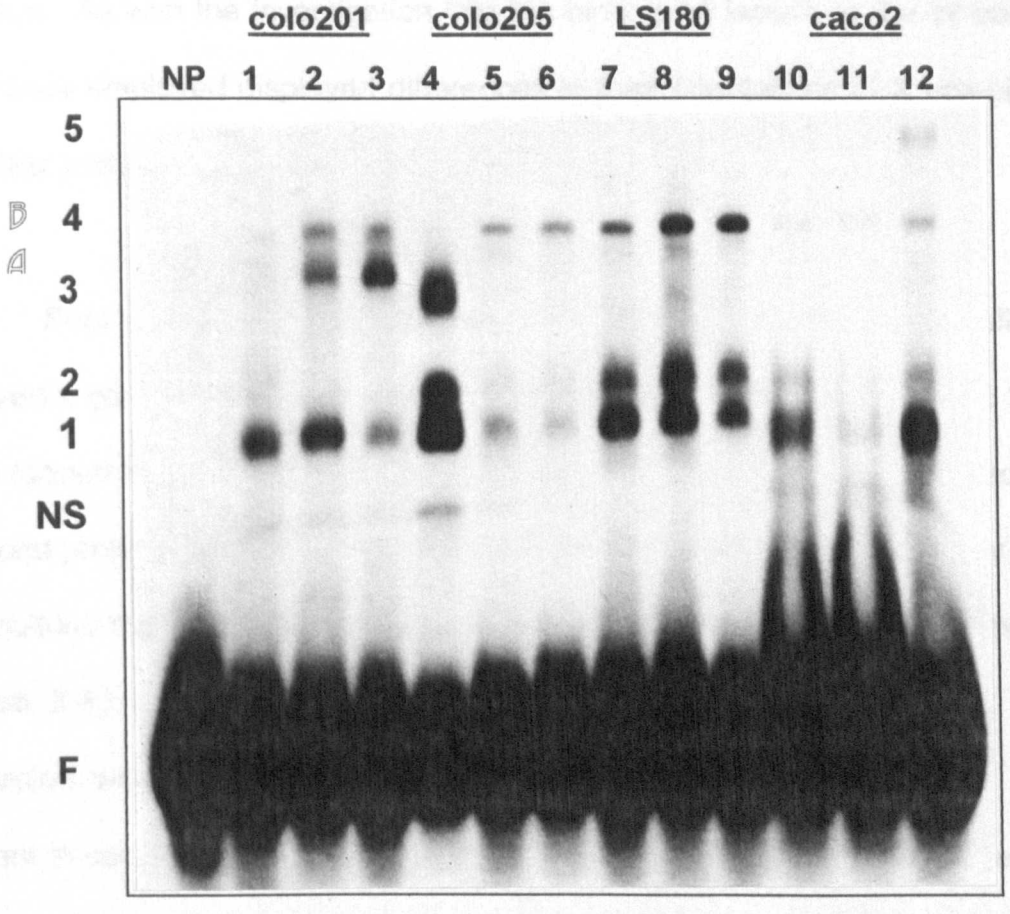
The faster migrating of the two novel bands (band A) did not have the same mobility as any band which was observed with the extracts from the other cell lines studied. The intensity of this band was greater at 24hr IFN-γ stimulation than at 6hr and this increase in intensity coincided with the reduced intensity of band 1 although the initial appearance of band A did not result in such a reduction in intensity of band 1. This suggested that the complex resulting in the appearance of band A was not formed by the acquisition of that protein(s) which caused the appearance of band 1.

Nuclear extracts from IFN-γ-treated colo 205 also contained "novel" X box-binding factors compared to the unstimulated control. The most prominent difference observed was the appearance of a band (band B) which had the same mobility as "band 5" (also present after IFN-γ treatment of colo 201). This

was coincident with the diminution of band 3 in extracts from cells treated for both 6 and 24hr. Treatment of colo 205 with IFN-γ also resulted in the disappearance of the complex which gave rise to band 2 in the unstimulated controls.

No differences were observed in the binding of nuclear proteins to the X box probe after IFN-γ-treatment of LS180, caco 2. The apparent appearance of the retarded complex 6 in caco 2 nuclear extracts is an artefact of the photographic procedure employed. This complex was observed in extracts from this cell line before and after IFN-γ stimulation although it is not clearly visible in figures 3.4 and 3.6.

Figure 3.6. Binding of nuclear factors to the HLA-DRα X box probe after treatment of cells with IFN-γ



Bandshift experiment employing nuclear extracts from unstimulated and IFN-γ-stimulated colorectal tumour cell lines colo 201, colo 205, LS180 and caco 2 (as indicated above lane markers) and a radiolabelled double stranded oligonucleotide probe corresponding to the HLA-DRα X box. Ten micrograms of nuclear extracts were incubated with 20,000 cpm of probe for 60min on ice prior to electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel at 150V for 3hr (see **section 2.11.3.** for method). Lanes 1, 4, 7 and 10: extracts from unstimulated cells. Lanes 2, 5, 8, and 11: cells treated with 1000U/ml IFN-γ for 6hr. Lanes 3, 6, 9 and 12: cells treated with 1000U/ml IFN-γ for 24hr. The free probe is indicated by "F"; NS=non-specific complex; NP=no protein control. Those binding complexes shown to be specifically bound are numbered according to their respective mobilities, bands A and B are induced in colo 201 after IFN-γ treatment.

3.3.3. Summary of results

The HLA-DRα X box probe was employed in a series of bandshift assays. As with the investigation into the binding of factors to the W box, the cell lines employed displayed differences in their populations of X box-binding nuclear proteins.

Prior to stimulation of cells with IFN-γ, the colorectal tumour cell lines shared a common, rapidly migrating complex (band 1 in figures 3.4 and 3.6.) - this formed the major complex in colo 201. In colo 205, LS180 and caco 2 a second protein complex, which migrated with marginally retarded mobility to the ubiquitous rapidly migrating complex, was shared. The next band (band 3 in figure 3.4.) was unique to uninduced colo 205, whereas a complex which migrated with slower mobility was observed only in LS180 and to a lesser extent in caco 2 nuclear extracts. A very retarded complex was observed only in nuclear extracts from caco 2 (band 6 in figure 3.6.).

The astrocytoma cell line U373MG contained four X box-binding complexes, two of which were the most abundant in this population.

No differences were observed in the binding of nuclear factors to the X box probe when LS180, caco 2 and U373MG had been treated with IFN-γ for 6 or 24hr. This observation was in contrast to those seen for IFN-γ-stimulated colo 201 and colo 205.

Interferon-γ-stimulated colo 201 contained two novel X box-binding complexes, the most retarded of which (band B) migrated with the same mobility as band 5 observed in uniduced and IFN-γ-stimulated LS180 and caco 2. The faster migrating of the two IFN-γ-induced complexes (band A) was not observed in nuclear extracts from any other cell line.

The stimulation of colo 205 by IFN-γ resulted in the loss of binding of the complexes which formed bands 2 and 3 in the uninduced state. Concurrent with this was the novel binding of a complex which migrated with the same mobility as band 5, again termed band B in figure 3.6.

3.4. Displacement of factors bound to the W box probe with unlabelled W and X box oligonucleotides

Competition bandshift assays were performed for two principal reasons. Firstly, the specificity of binding of the observed complexes was determined by "competing out" the binding with increasing molar excesses of unlabelled W box oligonucleotide in the bandshift reaction mixture. Secondly, the cross-reactivity of the complexes which bound to the W box could be assessed as regards their binding to the X box. The second use of competition bandshift assays came about from the observation that certain complexes which bound to the W box oligonucleotide probe migrated with similar if not the same mobility as factors which bound to the X box probe. Also, the sequence homology between the W and X boxes led to the possibility that certain proteins would have the ability to associate with either sequence element. By incorporating varying molar excesses of unlabelled X box oligonucleotide into the binding reaction prior to the addition of the radiolabelled W box probe, it was possible to determine whether any of the complexes which were observed bound to the W box could also bind to the X box; i.e. were factors "shared" by the two consensus elements?

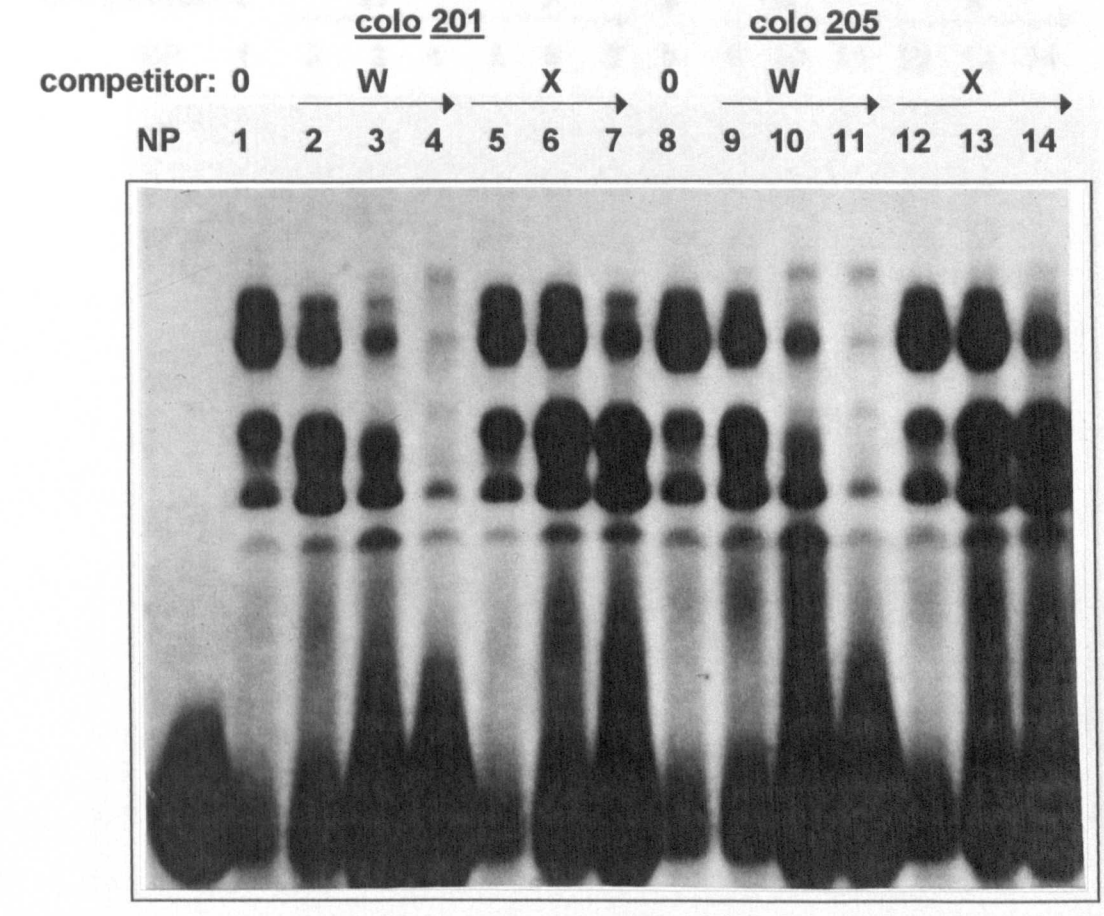
3.4.1. Specificity of binding of complexes to the W box probe

Since most of the complexes which were observed bound to the W box were only present in nuclear extracts from cells treated for 24hr with IFN-γ, competition bandshift assays were performed which employed such "24hr" extracts.

Nuclear proteins were preincubated at room temperature for 20min with carrier DNA, poly dI-dC:poly dI-dC and increasing amounts of unlabelled "competitor" oligonucleotide in molar excess from 1 to 500 times that of the probe. After addition of the probe, the reaction was incubated for a further 40-60min at room temperature prior to electrophoresis through a non-denaturing ploy acrylamide gel by the described method (see section **2.11.3**).

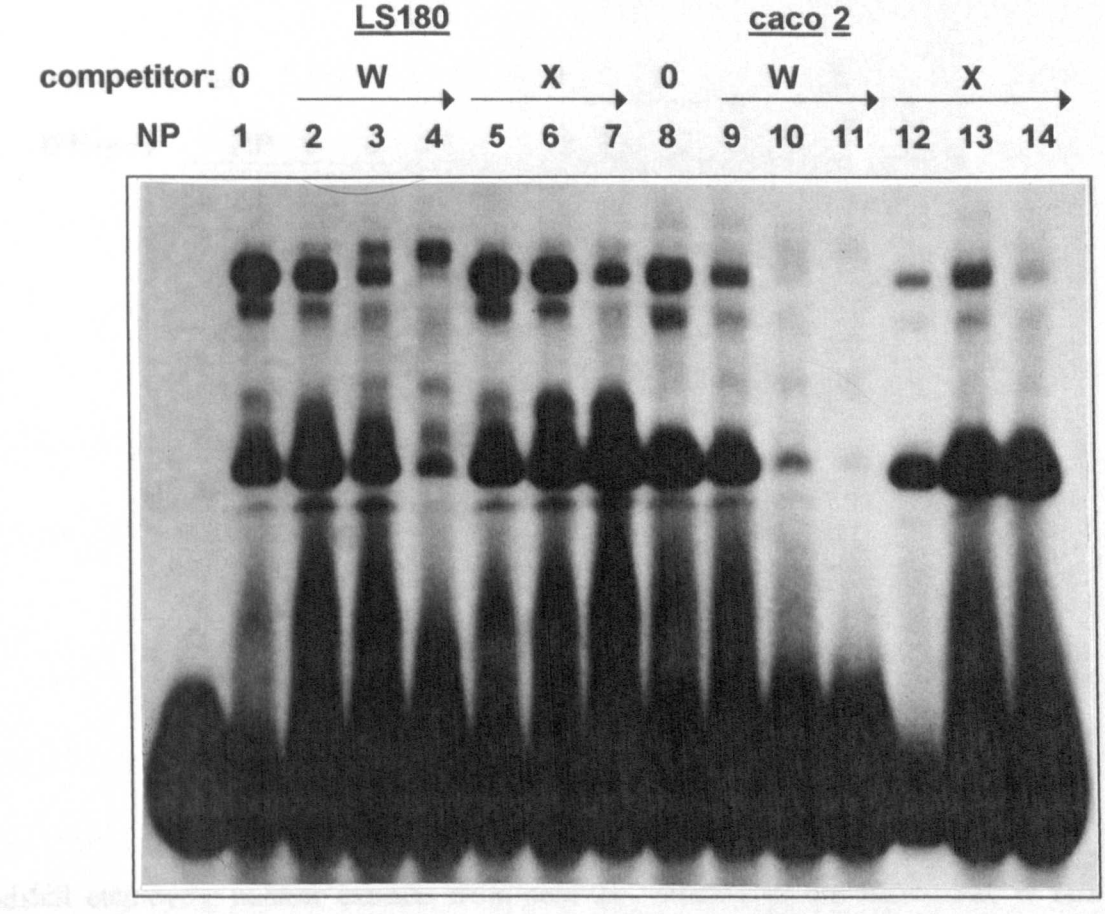
The results of a series of assays employing nuclear extracts from treated and untreated cells are shown in **figures 3.7.** to **3.8.** These results show that displacement of bound complexes started to occur with as little as an equal amount of unlabelled W box oligonucleotide, this was seen as the reduction in intensity of band 5 compared to the no competitor control with nuclear extracts from all four cell lines. All complexes corresponding to bands 2 to 5 were nearly fully competed out by 50X excess competitor. Such a competition pattern was indicative that these complexes were specifically bound to the W box probe.

Figure 3.7. Competition of HLA-DRα W box-bound factors from IFN-γ-induced cells by the W box and X box oligonucleotides



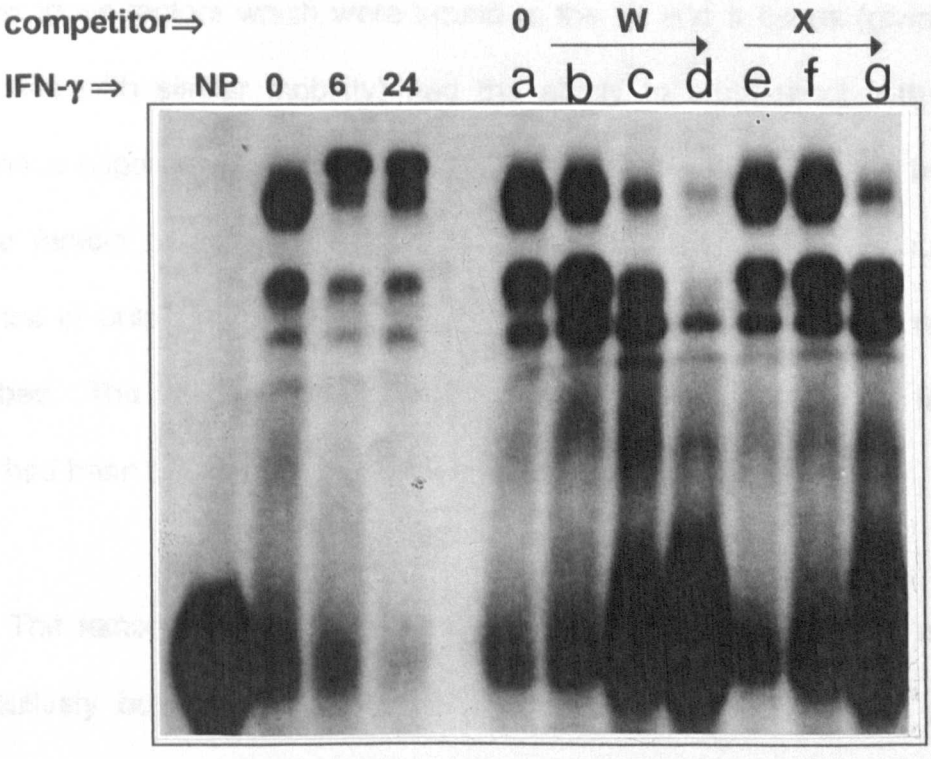
A) Bandshift with nuclear extracts form IFN-γ-stimulated colo 201 and colo 205 and the W box probe. Binding of factors has been competed out by the incorporation of 1X, 10X or 50X molar excess of unlabelled oligonucleotides corresponding to the W and X boxes (arrows indicate increasing competitor oligonucleotide concentration). Nuclear extracts (10μg), competitor and probe were incubated as described and DNA-binding moieties separated by electrophoresis through a 5% (w/v) polyacrylamide gel at 150V for 3hr.

Figure 3.7. (contd.)



B) Bandshift with nuclear extracts form IFN-γ-stimulated LS180 and caco 2 and the W box probe. Binding of factors has been competed out by the incorporation of 1X, 10X or 50X molar excess of unlabelled oligonucleotides corresponding to the W and X boxes (arrows indicate increasing competitor oligonucleotide concentration). Nuclear extracts (10μg), competitor and probe were incubated as described and DNA-binding moieties separated by electrophoresis through a 5% (w/v) polyacrylamide gel at 150V for 3hr.

Figure 3.8. Competition of HLA-DRα W box-bound factors from uninduced colo 201 by the W and X box oligonucleotides



Bandshift employing nuclear extracts from colo 201 binding to the HLA-DRα W box probe. 0, 6 and 24 indicate that cells were treated with (1000U/ml) IFN-γ for these times (in hours) prior to preparation of nuclear extracts. The binding of proteins from untreated cells to this probe was competed out by increasing concentrations (indicated by the horizontal arrow) of unlabelled W and X box oligonucleotides. The relative competitor oligonucleotide concentrations were: lane A: 0x; lanes B and E: 1x; lanes C and F: 10x; lanes D and G: 50x molar excess of the labelled W box probe. Nucleoprotein-DNA complexes were separated from the free probe by electrophoresis through a 5% (w/v) non-denaturing polyacrylamide gel for 3hr as described.

3.4.2. "Cross-reactivity" of W box-binding factors with the X box

Competition bandshift assays were performed in order to determine whether those factors which were bound to the W and X boxes (giving rise to complexes with similar mobility) had the ability to cross-react with the two consensus oligonucleotides. Assays were performed in which the binding of nuclear factors to the radiolabelled W box probe was competed out by the presence of unlabelled X box oligonucleotide in the preincubation mixture as described. The result of such an assay employing nuclear extracts from cells which had been treated with IFN-γ for 24hr is shown in **figure 3.7.**.

The factor(s) bound to the W box which gave rise to the least mobile constitutively bound complex in LS180 and caco 2 and the IFN-γ-induced complex in colo 201 and colo 205 was partially competed out by the presence of a 50X molar excess of unlabelled X box oligonucleotide. This implied a degree of cross reactivity for this factor although it was not competed out with the same readiness that was observed when the W box was used as competitor versus the W box probe; in that case a 10X molar excess of unlabelled oligonucleotide was sufficient for near total ablation of binding.

The same results were obtained for those factors from uninduced cells which bound to the W box probe. **Figure 3.8.** displays how those factors from uninduced colo 201 were competed out by both W and X box oligonucleotides. Most complexes were competed out by upto 50X molar excess of W box

oligonucleotide, yet only the most retarded band showed any degree of association with the X box.

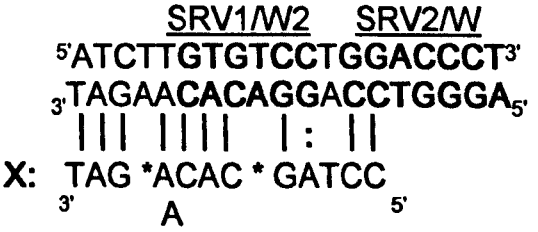
None of the other factors which bound to the W box either before or after IFN-γ-stimulation of cells were competed out by the presence of excess X box oligonucleotide.

3.4.3. Discussion

Competition bandshift assays were used to show which complexes bound to the HLA-DRα W box probe were specific for that consensus element. It was determined that 4 out of the 5 observed bands were due to the binding of complexes specific for the W box.

The cross reactivity of binding of W box-binding factors to the X box oligonucleotide was also studied. It was shown that although certain factors which bound to the W box might also be bound to the X box (through similarities in their migratory properties), only one W-binding factor had the ability to be partially sequestered by an unlabelled X box oligonucelotide. One W box-binding protein, termed W-B1 has also been shown to bind to the sequence within the W box corresponding to the most 5' part of this element (Tsang, *et al.*, 1990; Cogswell, *et al.*, 1990) which shows a strong inverted homology to the X box:

Homology between HLA-DRα W box and X box



Notes:
The W element is shown as dissected into two Servenius-like sequences, SRV1 and SRV2. The homologous X-box sequence is aligned beneath the W box. Solid lines between bases represent identical base pairs while dotted lines indicate a conserved base change.

3.5. Displacement of factors bound to the X box probe by unlabelled X and W box oligonucleotides

Similar competition bandshift experiments to those described for determining W box-binding specificity and cross reactivity were performed employing the X box probe. As with the W box, the reasons for such experiments were to determine whether those factors observed binding to the X box were specific for that element and to identify any factors which might also have the ability to interact with the W box.

3.5.1. Specificity of binding of factors associated with the X box probe

Competition assays were performed in the same manner as that described for the W box. An example of one such assay employing nuclear extracts from cells treated for 24hr with IFN-γ is shown in **figure 3.9.** This shows that most of the factors bound to X box were competed out by a 500X molar excess of unlabelled X box oligonucleotide. The only band which could not be competed out was band 1 and it was determined that this band was due to non-specific binding.

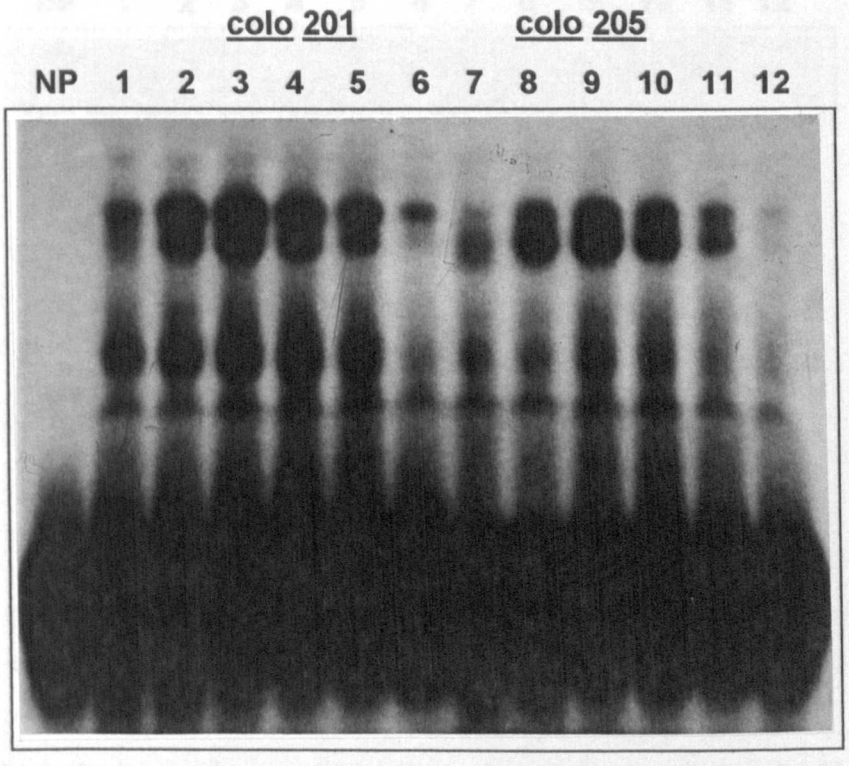
The concentration of competitor required for dissociation of bound factors was much higher than that required for the dissociation of factors from the W box probe when competed out by excess W box oligonucleotide: 500X molar excess X box compared to 10-50X molar excess of the W box (see

section **3.4.1.**). Such an effect was most likely to be due to the strong association of factors to this probe rather than the binding of factors being non-specific since the complexes were displaced by excess competitor eventually.

3.5.2. Cross-reactivity of X box-binding factors with the W box

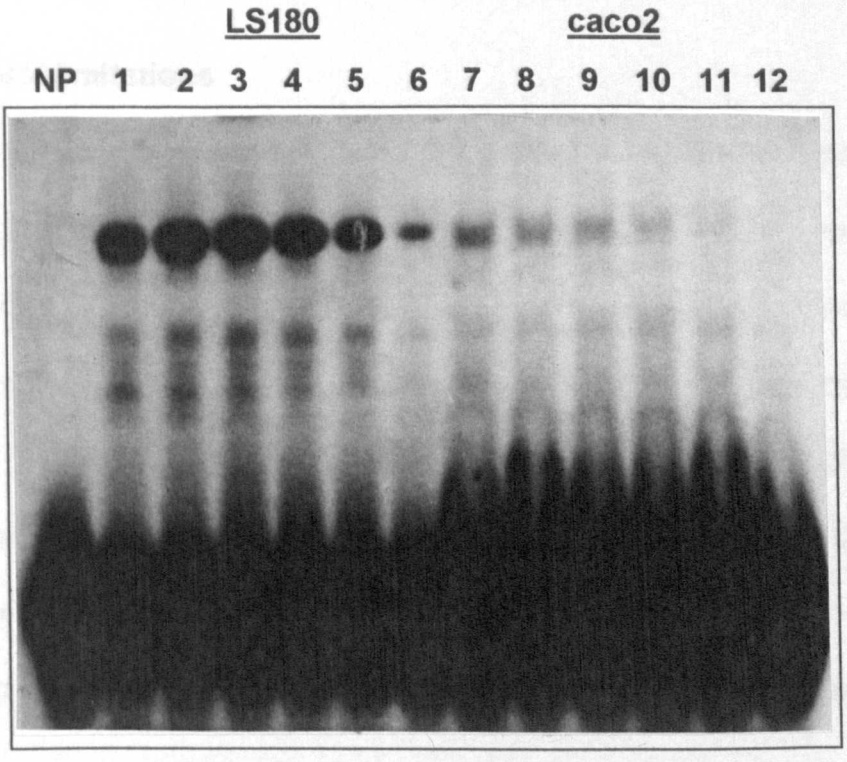
Cross-competition assays were also performed in which an attempt was made to compete out X box binding factors with varying molar excesses of the W box oligonucleotide. The protocol was followed as described employing nuclear extracts from cells treated with IFN-γ for 24hr and W box oligonucleotide was used in 10-500X molar excess of the X box probe. No cross-competition was observed under these circumstances. This result suggested that no factors which bound to the X box could bind to the W box. However, due to the high affinity with which factors were bound to the X box (as determined through competition assays), the inability of the W box to displace X box-binding factors was not unexpected.

Figure 3.9. Displacement of factors bound to the HLA-DRα X box probe by the X box oligonucleotide



A) Bandshift employing nuclear protein extracts from colo 201 and colo 205 which had been untreated prior to nuclear protein preparation. Ten micrograms of nuclear extracts were incubated with radiolabelled X box probe and increasing molar excesses of unlabelled X box oligonucleotide. The relative concentration of competitor employed was: Lanes 1 & 7: 0x; lanes 2 & 8: 1x; lanes 3 & 9: 10x; lanes 4 & 10: 50x; lanes 5 & 11: 100x; lanes 6 & 12: 500x molar excess of the probe. Protein-DNA complexes were separated from the free probe as described.

Figure 3.9. (contd.)



B) Bandshift employing nuclear protein extracts from LS180 and caco 2 which had been untreated prior to nuclear protein preparation. Ten micrograms of nuclear extracts were incubated with radiolabelled X box probe and increasing molar excesses of unlabelled X box oligonucleotide. The relative concentration of competitor employed was: Lanes 1 & 7: 0x; lanes 2 & 8: 1x; lanes 3 & 9: 10x; lanes 4 & 10: 50x; lanes 5 & 11: 100x; lanes 6 & 12: 500x molar excess of the probe. Protein-DNA complexes were separated from the free probe as described.

3.6. Discussion

Experimental limitations

Bandshift assays, also known as gel retardation and electromobility shift assays, proved useful in the detection of DNA binding proteins derived from the nuclei of human tumour cell lines. This type of technique is best employed for the detection of DNA binding factors specific for a particular sequence of DNA where the exact sequence of DNA to which binding occurs is not required. Detection of binding factors relies on the retarded electrophoretic progress of a radiolabelled probe through a non-denaturing polyacrylamide gel when nucleoprotein-DNA complexes are formed during the initial binding reaction.

The binding of proteins with different individual electrophoretic properties results in a number of bands being retarded compared to the progress of the free probe. Since non-denaturing polyacrylamide gels are employed to maintain the nucleoprotein-DNA complexes, proteins are separated according to their charge and mass. Hence it is inappropriate to assume that more retarded complexes contain "bigger" protein moieties compared to those complexes which migrate more freely.

This technique does not distinguish whether retarded bands are due to the binding of single or multiple protein factors to a particular probe molecule. As a consequence, it is not valid to assume that complexes with the same electrophoretic mobility represent the same nucleoprotein-DNA complexes. If it

could be assumed that complexes which have the same migratory properties are due to a single protein, the crudity of the technique could not determine the exact binding site.

With all of these considerations taken into account, when employed to its full potential, this type of technique affords a valuable means of analysing the populations of DNA binding proteins specific for a particular sequence of DNA under varying conditions. Indeed, for this part of the present investigation, these techniques, and variations thereof, proved ideal for the assessment of populations of HLA-DRα W and X box binding proteins in the cell lines studied.

Association of nuclear factors with the HLA-DRα W and X boxes.

The HLA-DRα W and X boxes were studied individually in order to correlate differences in surface expression of class II MHC antigen with differences in the binding of nuclear factors from unstimulated and IFN-γ treated cell lines to these consensus sequences. Indeed, specific for both of these *cis*-acting regulatory elements, the cell lines studied contained different populations of nuclear DNA binding proteins. Differences were also observed in the binding of nuclear factors to the W box probe after treatment of the HLA-DRα-inducible cell lines colo 201, colo 205 and U373MG with IFN-γ. Similarly for the X box, IFN-γ treatment of colo 201 and colo 205 led to changes in the array of nuclear-localised factors which associated with this probe.

Other researchers employing similar techniques with lymphoid cell lines such as Raji and Jurkat, and the epithelial cell line HeLa have described W and X box binding complexes. The identities of the factors which were shown to bind to these probes in the current investigation could not be determined and these binding activities could not be correlated with previously identified factors. Chapter 8 discusses the putative rôles for the W and X box-binding factors described in this study and how these may relate to those transcription factors already known to associate with the *cis*-acting sequences in other cell lines.

CHAPTER 4

CHAPTER 4: INVESTIGATION OF THE INTERACTION OF NUCLEAR DNA-BINDING PROTEINS TO THE HLA-DRα PROXIMAL PROMOTER

4.1. Introduction

The HLA-DRα proximal promoter region, defined as the 300 to 400 base pairs immediately prior to the transcriptional start site, was studied in order that the interaction of DNA-binding proteins with the promoter and with other such factors could be assessed. An attempt was made to translate the effects observed regarding the binding of nuclear proteins to the consensus W and X box probes (see chapter 3) to the whole promoter region scenario. The employment of promoter fragments as radiolabelled probes was intended to enable the "footprinting" of the region described in order that binding sites of individual transcription factors could be determined (described in chapter 5). However, such footprinting probes proved to be of greater use in the bandshift experiments documented in the current chapter.

The primary objective of the experimentation described here was to show that the "induced" binding of novel factors to the W and X boxes upon interferon-γ treatment of cells was also reflected at the level of the whole promoter. The cooperation of factors towards their interaction with the promoter was assessed by adapting the competition bandshift assay techniques described in chapter 3.

4.1.1. Experimental considerations

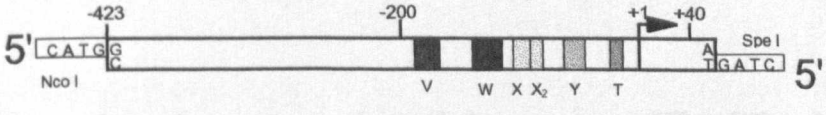
Radiolabelled double-stranded DNA fragment probes corresponding to regions of the proximal promoter were employed in bandshift assays with nuclear extracts from the cell lines under investigation. The DNA fragments used for the preparation of probes were derived from a larger 680bp HLA-DRα promoter fragment which had been cloned into the Bam HI restriction site in polylinker of the phagemid pBluescript $(KS)^{+}$. The probes employed in the majority of experiments described in this chapter contained the consensus upstream sequences V, W, X, Y and the TAATA box; the exception being the DRα370 probe which lacked the TAATA element. They were end-labelled on one strand by end-filling the 3' recesses created by restriction endonuclease digestion with [α-^{32}P] dNTPs in reactions catalysed by AMV reverse transcriptase as described in section **2.11.2.**

Due to the increased lengths of probes compared to the oligonucleotides employed in other assays, non-specific binding of proteins to the probes was much greater. This was counteracted by the addition of greater concentrations of poly (dI-dC):poly (dI-dC) to the incubation mixtures as determined in preliminary titration experiments. The amount of nuclear extract incorporated into the binding reactions was also titrated for each probe employed such that non-specific binding was minimised.

4.1.2. The HLA-DRα 470 probes

The probe which was most commonly employed for use in this type of bandshift assay was one which covered 470bp of sequence corresponding to the proximal promoter and a portion of the 5' untranslated region of the HLA-DRα gene. The sequence spanned the region of the promoter from nucleotides -423 to +47 relative to the start of transcription (cap site). Within this region, the defined consensus upstream sequences of the promoter were present in their native locations as described (see **figure 4.1** below). This fragment was generated by restriction endonuclease digestion of the larger HLA-DRα 680 fragment which had been amplified by PCR and cloned into the polylinker of pBS $(KS)^+$. Excision of the fragment was always performed in preparation for and during the radiolabelling of probes as described in section **2.11.2.** The order in which the restriction enzymes (Nco I and Spe I) were employed was dependent on whether the resultant probe was to be labelled on the coding or non-coding strand.

Figure 4.1. The HLA-DRα 470 fragment

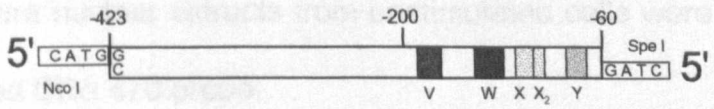


Notes:
The figure is a schematic representation of the HLA-DRα 470 base pair promoter fragment employed in bandshift and footprinting assays following radioactive labelling with [α-^{32}P] nucleoside triphosphates. The consensus upstream sequences of the promoter are indicated by shaded boxes, their respective names are indicated beneath; the TAATA box is referred to by "T". The scale is shown by numbered nucleotides. The restriction endonucleases employed to generate this fragment and the nucleotide sequences of the respective recognition sites are shown. The site of the start of transcription is shown by an arrow.

4.1.3. The HLA-DRα 370 probe

Occasionally, a truncated promoter fragment probe was employed in bandshift assays. This probe, termed HLA-DRα 370, was derived from the plasmid pBS (KS)$^{+}$DRα582 and was the same as the HLA-DRα 470 probe except that the 3' most 100 base pairs had been removed by PCR prior to its cloning into the vector. The resulting fragment probe corresponded to the region of the promoter from bases -423 to -53 (relative to the cap site) and contained all the upstream consensus elements of the HLA-DRα promoter except the TATA box. The use of this probe in assays was intended to aid the clarification of any bandshifts where there may have been confusion due the presence of TATA box-binding factors. A schematic representation of the HLA-DRα 370 probe is shown in **figure 4.2.**

Figure 4.2. The HLA-DRα 370 fragment



Notes:
The figure is a schematic representation of the truncated HLA-DRα 370 base pair promoter fragment employed in bandshift and footprinting assays following radioactive labelling with [α-^{32}P] nucleoside triphosphates. The consensus upstream sequences of the promoter are indicated by shaded boxes, their respective names are indicated beneath.. The scale is shown by numbered nucleotides. The restriction endonucleases employed to generate this fragment and the nucleotide sequences of the respective recognition sites are shown.

4.2. Binding of nuclear factors to the HLA-DRα 470 probe

4.2.1. Constitutive binding of nuclear proteins

The presence of constitutive HLA-DRα promoter-binding factors in the nuclear extracts of the colorectal cell lines colo 205, LS180 and caco 2 was assessed by bandshift assay employing the 470bp probe. The number of cell lines chosen was restricted because of the intensive and time consuming nature of this experimentation. The colorectal tumour cell line colo 201 was excluded due to its similar properties to colo 205. U373MG were excluded due to the repeated low concentrations of nuclear extracts obtained during protein preparations.

The constitutive binding of factors to this promoter fragment was defined as that which was observed with nuclear extracts from cells which had been untreated with cytokines. **Figure 4.3.** shows the result of a bandshift experiment where nuclear extracts from unstimulated cells were incubated with the radiolabelled DRα 470 probe.

The binding of factors to individual upstream consensus elements was competed out by the incorporation of excesses of oligonucleotides corresponding to the W, X and Y boxes in a "pair-wise" manner such that Y & X, W & Y, and X & W box binding factors were competed out sequentially. In such a system, addition of excesses of both X and Y box oligonucleotides to the binding reaction mixture would not permit association of X or Y box-specific

factors to the probe, but binding of proteins to the W box (and other promoter elements) could occur. By employing all combinations of competitor oligonucleotides, complexes observed binding to the promoter probe could be attributed sequentially to the individual sequence elements.

i) Binding of nuclear factors from untreated colo 205

Nuclear extracts from unstimulated colo 205s provided three major complexes which bound to this probe (see **figure 4.3.**). In the absence of competitor oligonucleotides, the majority of binding was observed as two retarded complexes which migrated with slow mobility compared to the free probe.

The complex which gave rise to the most retarded band was effectively competed out by the presence of excess X box oligonucleotide. When the W and Y box oligonucleotides were present in excess, this band was present with the same intensity as observed where no competitor was present. Such evidence was indicative that this factor was bound specifically to the X box.

When the binding of complexes to this probe was competed out by the combined excess of X and Y box oligonucleotides, the most retarded band observed in the absence of competitor was resolved as being attributed to the binding of more than one protein factor. This was manifested as the resolution of a W box binding factor which was obscured prior to the removal of other complexes by competition with X and Y box oligonucleotides.

The band described as band 2 was present regardless of incorporation of competitor, however, its appearance was most abundant where the W and Y box oligonucleotides had been included in the binding reaction mixture. The least retarded band which was attributed to the binding of a nuclear protein(s)

(band 1) was not competed out by any of the combinations of oligonucleotides employed in this assay. It was also observed with the same intensity regardless of this treatment. Such a result led to the assumption that this band was not caused by the binding of a protein(s) to the probe in the location of the W, X or Y boxes or a consensus element with sequence homology to these sequences.

ii) Binding of nuclear factors from untreated LS180

With nuclear extracts from unstimulated LS180, the binding of factors to this probe was different to that observed with the colo 205 extracts. **Figure 4.4.** shows the result of one such bandshift. Firstly, the most retarded band observed with the colo 205 nuclear proteins was only faintly visible in this case and here it was resolved into its two components. Also, the least retarded band (band 1) was similarly only faintly present in this situation. A novel binding factor was also observed with these nuclear extracts. This gave rise to a faint band which migrated slightly faster than "band 2" in colo 205.

Upon the incorporation of the combined competitors into the binding reaction mixture, band 1 was observed with greater intensity. The binding of other complexes from the LS180 extracts to this probe was unaltered by the presence of competitor oligonucleotides.

iii) Binding of nuclear factors from untreated caco 2

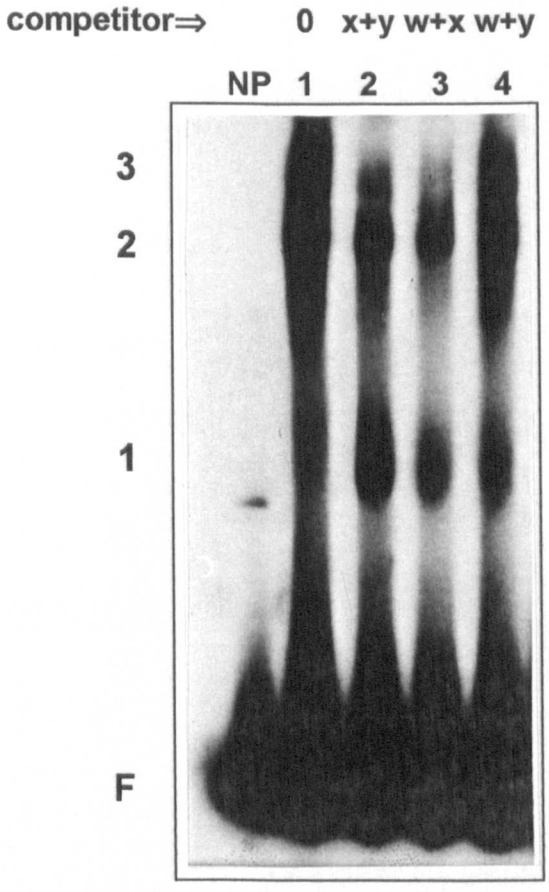
Nuclear extracts from unstimulated caco 2 contained HLA-DRα promoter-binding proteins which resulted in the observation of three gel retardation complexes with the 470bp probe (refer to **fig. 4.5.**). The complexes observed were the same as the three most retarded complexes binding to this probe from unstimulated colo 205. However, association of proteins in the formation of the slowest migrating complex was much less than observed with colo 205 nuclear extracts and its presence was as a minor complex. This was the opposite to with colo 205 where this protein's rôle in the in the formation of nucleoprotein complexes with the HLA-DRα 470 probe was a major one.

The slowest migrating band was effectively competed out by the addition of excess W, X and Y box oligonucleotide indicating either a weak or non-specific association of this factor with the probe. The corresponding band observed with colo 205 nuclear extracts was shown to be due to the specific interaction of a factor(s) with the X box region of the promoter. It was therefore unlikely that the same band observed with caco 2 extracts was due to the non-specific association of a factor with the promoter, rather, its binding was specific to the X box region but the association was a weak one which allowed its displacement from the probe by X box-related sequences.

Another difference observed between the binding of nuclear proteins from caco 2 and those from other cell lines was the absence of the fast migrating complex termed "band 1" with colo 205 and LS180. This complex was

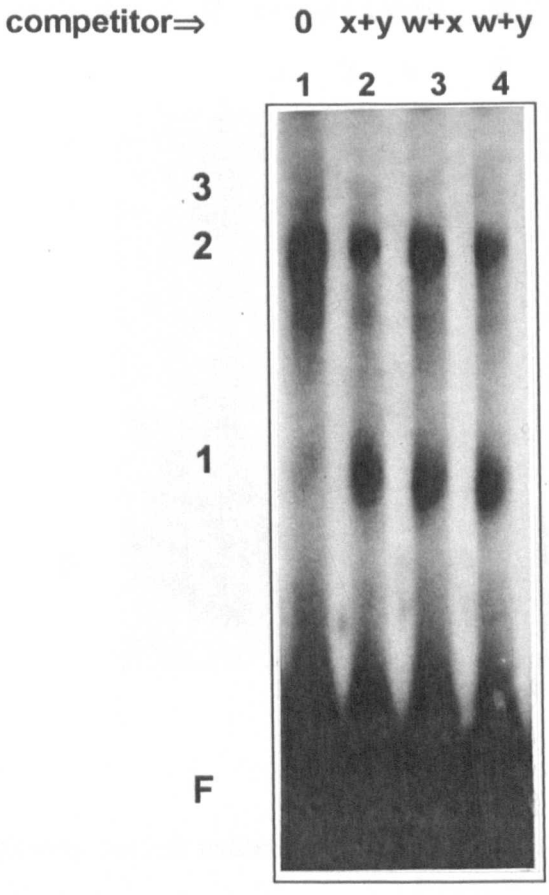
not observed in extracts from unstimulated caco 2 and its presence was not altered by the incorporation of competitor oligonucleotides - a phenomenon encountered when nuclear extracts from LS180 were employed under the same circumstances.

Figure 4.3. Binding of unstimulated colo 205 nuclear proteins to the HLA-DRα 470 probe



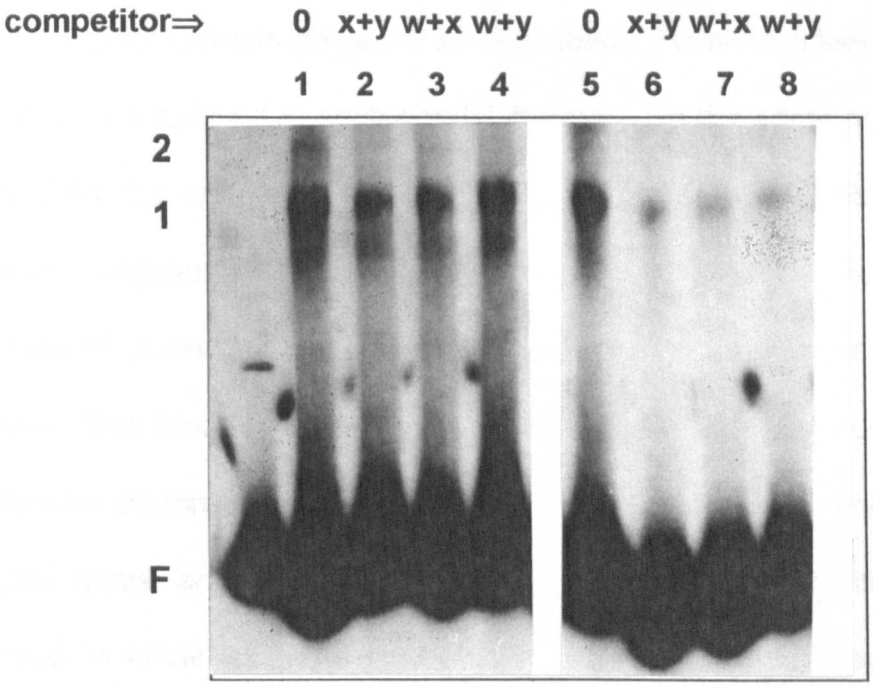
Bandshift employing nuclear extracts from unstimulated colo 205 and the HLA-DRα470 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text).

Figure 4.4. Binding of unstimulated LS180 nuclear proteins to the HLA-DRα 470 probe



Bandshift employing nuclear extracts from unstimulated LS180 and the HLA-DRα470 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text).

Figure 4.5. Binding of unstimulated and IFN-γ-stimulated caco 2 nuclear proteins to the HLA-DRα 470 probe



Bandshift employing nuclear extracts from unstimulated and IFN-γ-stimulated caco 2 and the HLA-DRα470 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text). Lanes 1-4: untreated nuclear extracts; lanes 5-8: lanes from caco 2 treated with IFN-γ (1000U/ml) for 24hr.

4.2.2. Binding of nuclear factors to the HLA-DRα 470 probe after interferon-γ-treatment of cells

Cells were treated with 1000U/ml recombinant human IFN-γ for 6 and 24hr and nuclear extracts prepared as described. Seven to fifteen micrograms of extract was employed in each bandshift assay. In the same manner to that described for the constitutive binding of factors to the HLA-DRα 470 probe, competitor oligonucleotides corresponding to the consensus upstream sequences W, X and Y were incorporated in a pair-wise manner into selected reactions. The binding reactions for untreated and IFN-γ stimulated nuclear extracts were performed at the same time with the aliquots of extracts prepared from cells grown and harvested simultaneously in order that differences and similarities in binding could be assessed. The result of assays employing nuclear extracts from IFN-γ-treated cells is shown in **Figures 4.6.-4.7.**

i) Binding of nuclear factors from IFN-γ-treated colo 205

Treatment of this cell line with 1000U/ml IFN-γ for 6hr prior to harvesting and extraction of nuclear proteins revealed the increased overall binding of protein factor(s) to this probe (see **figure 4.6a**). This was manifested in the reduced mobility of the slowest migrating complex observed prior to IFN-γ treatment. In the study of constitutively bound HLA-DRα promoter binding factors, it was determined, by competition studies, that this band was due to the binding of ubiquitous complexes to the W and X boxes. It was therefore determined that the increased binding observed after 6hr IFN-γ treatment was due to increased interaction at the W/X box domain of the promoter or with

other proteins already associated with the promoter at these sites. It was concluded that the reduced mobility IFN-γ-induced binding moiety was due to increased association with factors already bound at these locations due to the reduced intensity of the W/X band in proportion to the appearance of the novel band.

The treatment of colo 205 cells with 1000U/ml IFN-γ for 24hr prior to their harvesting and extraction of nuclear proteins led to a different pattern of binding of complexes to the HLA-DRα 470 probe compared to the unstimulated controls with the result that net binding of proteins was reduced (see **figure 4.6.b**). This was manifested in the association of complexes with the probe resulting in one major band corresponding to the second most retarded band observed with extracts from unstimulated colo 205s. A diffuse band which migrated marginally faster than this band was also observed.

Stimulation of cells with IFN-γ for 24hr also resulted in the loss of binding of the factor contained in colo 205 extracts which migrated with fastest mobility.

No differences were observed upon incorporation of competitor oligonucleotides into the binding reaction mixtures although the intensity of the most retarded band was increased upon competing out W and Y box-binding factors.

ii) Binding of nuclear factors from IFN-γ-treated LS180.

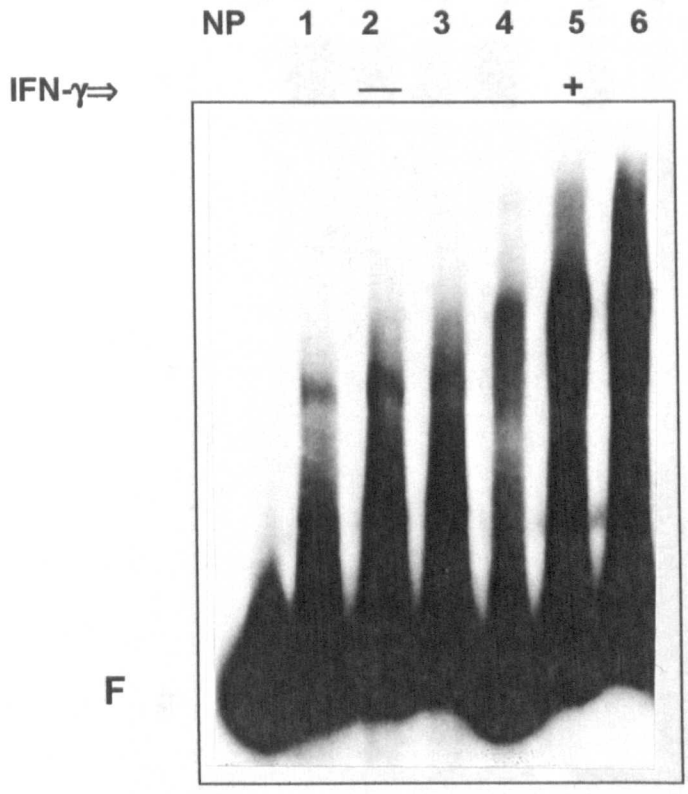
Prior to the incorporation of competitor oligonucleotides into the reaction mixture, the nuclear extracts from IFN-γ-stimulated LS180 contained factors which bound to the HLA-DRα 470 probe resulting in the appearance of one major band (refer to **figure 4.7.**). This band had the same mobility as that band which was observed after binding of IFN-γ-stimulated colo 205 nuclear extracts.

When the W, X and Y box-binding factors were competed-out in the pair-wise manner described, the fastest migrating band which was observed with extracts from unstimulated colo 205 and LS180 (but not with IFN-γ-stimulated colo 205) also became apparent. This band was as abundant as the slower migrating complex observed prior to competitor incorporation and did not appear to be due to the specific association of a protein factor with any of the consensus elements W, X or Y. Such a conclusion was made due to the fact that band intensity did not change with the addition of any combination of competitor oligonucleotides.

iii) Binding of nuclear factors from IFN-γ-treated caco 2

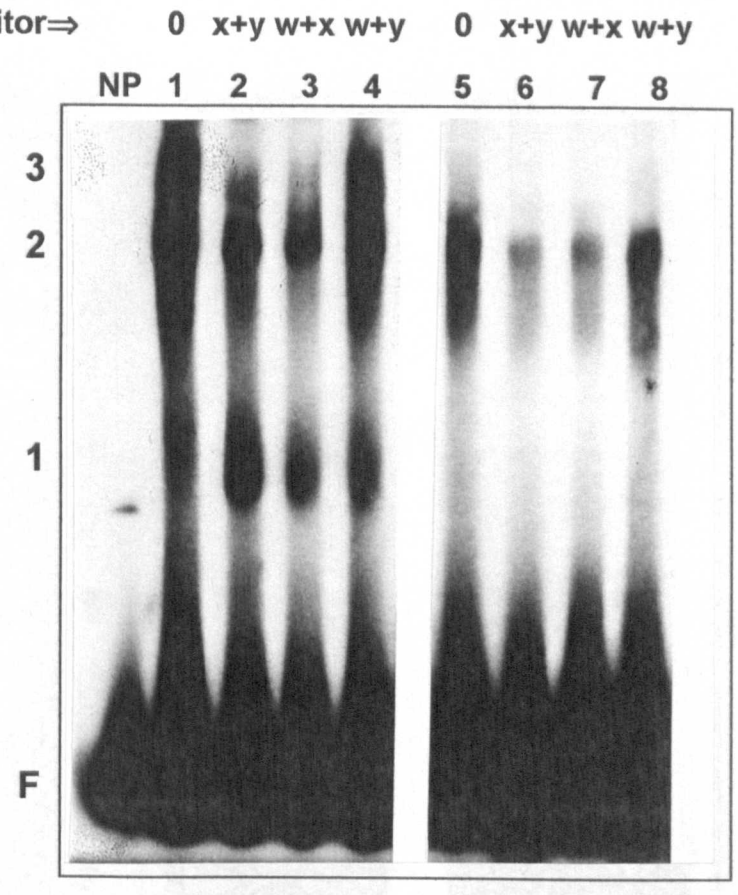
There was no net difference in binding observed when caco 2 were treated with IFN-γ. Complexes bound to the probe to yield two major bands which corresponded to bands 1 and 2 in **figure 4.5.** When the competitor oligonucleotides were added as described, the result was the near total disappearance of band 1 and a high degree of dissociation of factors causing the observed band 2 (see **figure 4.5.**).

Figure 4.6. Binding of IFN-γ-stimulated colo 205 nuclear factors to the HLA-DRα 470 probe



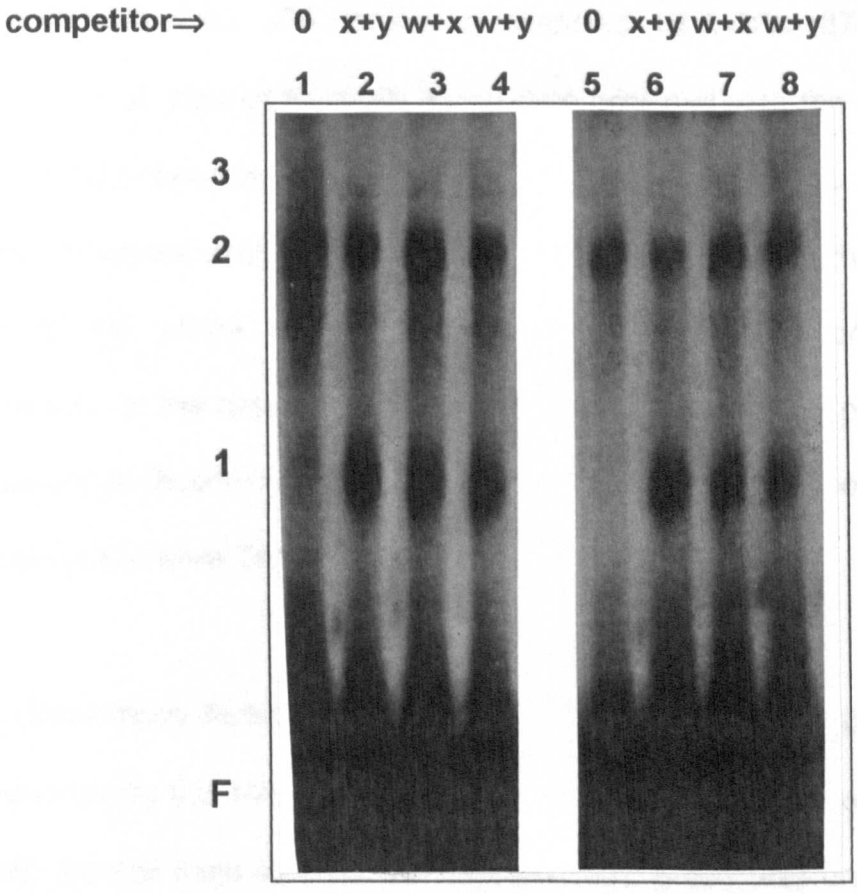
Bandshift with increasing concentrations of colo 205 nuclear extracts binding to the HLA-DRα470 probe. Five to fifteen micrograms of nuclear extract was incubated with probe on ice and nucleoprotein-DNA complexes separated from free probe by electrophoresis as described. Lanes 1 & 4: 5μg; lanes 2 & 5: 10μg; lanes 3 & 6: 15μg nuclear protein extracts. Nuclear proteins used in lanes 4 to 6 were prepared from cells which had been treated with IFN-γ (1000U/ml) for 6hr.

Figure 4.6. (contd.)



B) Bandshift employing nuclear extracts from unstimulated and IFN-γ-stimulated colo 205 and the HLA-DRα470 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text). Lanes 1-4: untreated nuclear extracts; lanes 5-8: nuclear extracts from cells treated with IFN-γ (1000U/ml) for 24hr.

Figure 4.7. Binding of IFN-γ-stimulated LS180 nuclear factors to the HLA-DRα 470 probe



Bandshift employing nuclear extracts from unstimulated and IFN-γ-stimulated LS180 and the HLA-DRα470 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text). Lanes 1-4: untreated nuclear extracts; lanes 5-8: nuclear extracts from cells treated with IFN-γ (1000U/ml) for 24hr.

4.2.3. Binding of nuclear factors from untreated and IFN-γ-stimulated colo 205 to the truncated HLA-DRα 370 promoter probe

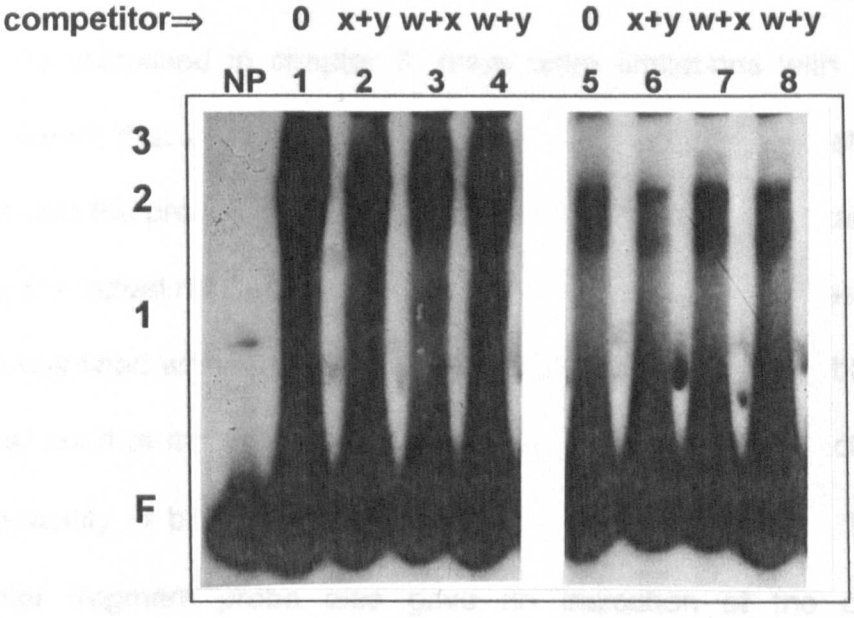
A study of the binding of nuclear factors from untreated and IFN-γ-stimulated colo 205 to the 3'-truncated HLA-DRα 370 probe was performed in an attempt to clarify those data obtained with the HLA-DRα 470 probe. It was possible that the bandshifts obtained using the 470 base pair promoter fragment probe showed the binding of factors to the TAATA box region of the probe and that these complexes were complicating the interpretation of the data. The removal of the TAATA box region of the probe was devised to determine if any of those "non-specific" factors bound to the 470 base pair probe were TAATA-associated.

Since those factors which bound to the HLA-DRα 470 probe - but not competed out by the consensus W, X and Y oligonucleotides - were ubiquitous between the cell lines studied, the HLA-DRα 370 probe was only studied with nuclear extracts from colo 205. It was assumed that the results of such experiments would be mirrored with nuclear proteins from the other cell lines so that repeated experiments employing limited stocks of these nuclear extracts were avoided.

The result of a bandshift employing this probe and untreated/IFN-γ stimulated colo 205 nuclear extracts is shown in **figure 4.8.** This result is the same as those others observed under the same conditions with the longer probe. This confirmed that the "non-specific" factors which bound to the

HLA-DRα 470 promoter probe were not associated with the region downstream from the Y box.

Figure 4.8. Binding of nuclear factors from unstimulated and IFN-γ-treated colo 205 to the HLA-DRα 370 probe



Bandshift employing nuclear extracts from unstimulated and IFN-γ-stimulated colo 205 and the HLA-DRα370 probe. Ten micrograms of nuclear extract were incubated with probe (and competitor oligonucleotides) for 1hr on ice before electrophoresis through 5% (w/v) non-denaturing polyacrylamide for 5hr. Competitor oligonucleotides were those corresponding to the HLA-DRα W, X and Y boxes, they were added in a pair-wise manner such that their incorporation would reveal the net binding of proteins at one site (for example, W + Y box competition would reveal the net binding at the X box, refer to text). Lanes 1-4: untreated nuclear extracts; lanes 5-8: nuclear extracts from cells treated with IFN-γ (1000U/ml) for 24hr.

4.3. Discussion

The bandshift experiments described in this chapter were of use in furthering the understanding of the interaction of DNA binding proteins with the HLA-DRα promoter.

As discussed in chapter 3, there were limitations with this technique which meant that a clear and definitive picture of the association of nuclear factors with the promoter fragment probes employed was not attainable. That is to say, the actual nucleotides with which binding factors were associated could not be identified with this type of assay. However, it was possible to determine whether each of the cell lines employed possessed nuclear factors which had the capability of binding to the HLA-DRα proximal promoter. The use of the promoter fragment probe also gave an indication of the dependency of factor-binding upon the presence of other *cis*-acting elements and the proteins which they bound.

It was noted that the results obtained from bandshift assays with the HLA-DRα promoter fragment probe indicated a somewhat simplified association of transcription factors compared to what might have been expected from those assays employing oligonucleotide probes. If the binding patterns from oligonucleotides were simply additive when resolved in the whole promoter situation, then it might be expected that twelve or more bands would appear with a promoter fragment probe. This was not the observation and it was

perhaps the case that the bands obtained with the promoter fragment probe were due to series of alternative binding "combinations" of those factors which associated with the probe. For example, slowly migrating complexes could have been due three or more proteins being bound, and high-mobility bands could have been attributed to single proteins associating with the probe. Such speculation cannot explain fully why the bandshifts discussed in this chapter seemed simple.

The putative rôles for the interaction of DNA binding proteins with the HLA-DRα promoter probes are discussed in Chapter 8.

CHAPTER 5

CHAPTER 5: LOCALISATION OF BINDING SITES OF TRANSCRIPTION FACTORS BY DNASE I FOOTPRINTING OF THE HLA-DRα 470 PROBE

5.1. Introduction

5.1.1. General comments

The clarification of the data obtained from the bandshift assays, such that individual binding sites of HLA-DRα promoter-binding proteins could be determined, formed the next part of these investigations. DNase I footprinting was employed in a variety of assays in an endeavour to furnish this requirement.

The cell lines discussed previously were employed in this investigation. Again, prior to harvesting of cells and extraction of nuclear proteins, they were treated with IFN-γ, or IFN-γ and IFN-αβ concurrently for 24hr, or remained unstimulated. Costimulation with IFN-αβ was performed in order to determine the effects, if any, of this cytokine upon the binding of nuclear proteins to the HLA-DRα promoter probe.

5.1.2. Protocol requirements and assay optimisation

The requirements for footprinting were similar to those for bandshift assays employing fragment DNA probes, but the reactions were "scaled-up" such that all the probe molecules were "covered" with protein. One hundred to 250μg of concentrated nuclear extracts (approximately $10\mu g\mu l^{-1}$) were incubated

with fmol amounts of high specific activity radioactive probes to achieve this.

The binding reaction mixture was also optimised in order to allow maximal binding of the majority of factors which are present in the crude nuclear protein extracts. The concentrations of divalent metal ions and salt were altered in accordance with optimal binding activity. The concentration of DNase I and the digestion conditions were optimised such that, on average, one incision was made per probe molecule. The most commonly used footprinting parameters employed in this study are detailed in section **2.11.5.**

Important in the interpretation of footprints were the inclusion of controls. Firstly, "no protein" controls were incorporated into each procedure such that comparisons could be made between those footprints obtained from the binding of proteins to the probe with the naked "DNA ladder" obtained from the partial digestion of probe alone.

In order that any areas of protection observed from the footprints could be localised within the probe, the chemically sequenced DNA ladder was also electrophoresed alongside the footprinting reactions. Maxam and Gilbert sequencing was employed since this method did not require "priming" of the probe and hence, the probe's length was not altered by the sequencing procedure. When the sequencing fragments were electrophoresed alongside those probe fragments obtained from DNase I digestion, the sequence of any region of the DNase I footprint could be deduced from the associated sequence.

5.2. DNase I footprinting of the HLA-DRα 470 probe in the presence of nuclear factors from unstimulated and IFN-γ and IFN-γ + IFN-αβ- treated colo 205, LS180 and caco2

Data from bandshift assays of this probe and the individual consensus upstream sequences W and X had revealed a number of differences in the apparent array of HLA-DRα promoter binding proteins contained within these cell lines under the stimulus of IFN-γ and with the untreated cells. These differences were obvious between the cell lines, but a marked difference in the binding (to the oligonucleotide probes W and X) of nuclear proteins from colo 205 nuclei was also observed after treatment of these cells with IFN-γ. The main objective of this part of the investigation was to determine whether the location of contact-points of these observed binding moieties were the same for each of those observed for the individual cell lines. It was also of interest to determine whether the "footprints" for those binding activities which appeared to be shared between cell lines and consensus upstream sequences were the same - indicating that the protein was the same.

It had been argued that the protein(s) giving rise to a binding activity of the same or similar migratory properties might be active in one cell line yet of no transcriptional potential in another - differences in the footprint of the transcription factor might have resulted from this and the hypothesis been verified.

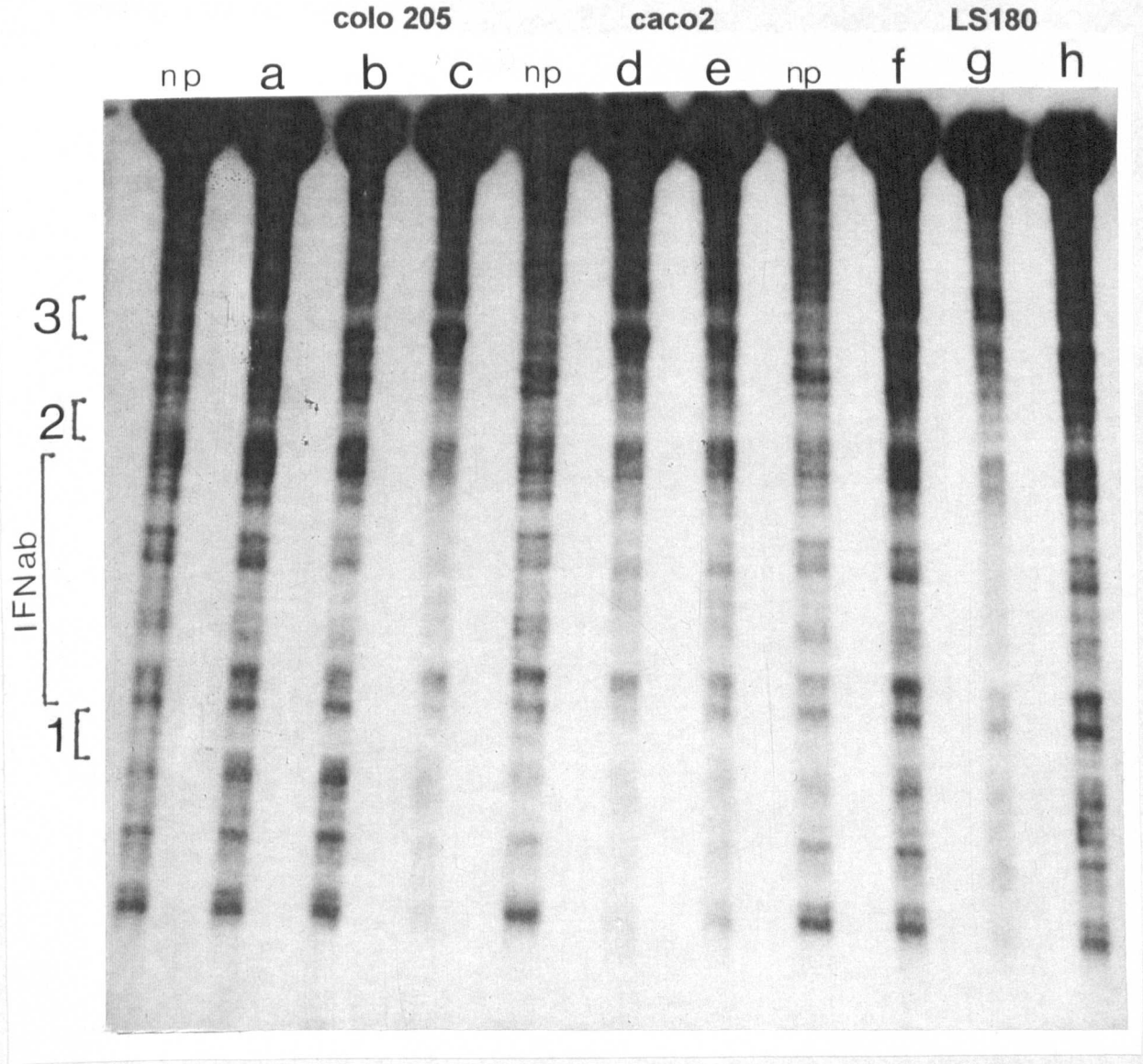
The appearance of novel W and X box-binding activities in the nuclear extracts from IFN-γ treated colo 205 (and colo 201) compared to those cells which had been untreated, led to the question of whether this/these "induced" complexes were due to: a) the binding of a novel factor to these sequence elements; b) the binding of an IFN-γ-induced protein 'cofactor' to one or more of those proteins already bound to the *cis*-element (without itself associating with the DNA); c) the modification of a constitutively-bound factor(s) upon stimulation of the cells with IFN-γ. Footprinting techniques may have allowed the interpretation of the corresponding bandshift data in this respect.

Concurrent with this was the study into the effects of simultaneous treatment of cells with IFN-γ and IFN-αβ. The physiological effects of such treatment upon the stimulation of MHC class II surface expression is discussed in Chapters 6 and 7. However, this study was undertaken as a "prequel" and as a complement to that which is described later.

The result of a footprint assay employing the HLA-DRα 470 probe (labelled on the coding strand) and nuclear protein extracts from the respective cell lines before and after cytokine treatment is shown in **figure 5.1.**

Figure 5.1. The HLA-DRα470 probe labelled on the coding strand was digested by 1U (1μl) of bovine pancreas DNase I for 2min at room temperature. Lanes labelled "NP" indicate that the probe was digested in the absence of nuclear proteins. Other lanes (a-h): the probe was digested under the same conditions, but after incubation with 100μg of nuclear protein extracts from the cell lines indicated above the lanes. Cells were untreated (lanes a, d and f) or treated with 200U/ml IFN-γ (lanes b, e and h) or 200U/ml IFN-γ + 500U/ml IFN-αβ (lanes c and g). Regions of protection are indicated at the left hand side of the figure. Protected regions 1, 2 and 3 were observed constitutively, whereas that region labelled "IFN-ab" was observed only after the concurrent treatment of cells with IFN-γ and IFN-αβ. The sequences of the protected regions were determined from the Maxam & Gilbert sequence of the same probe and these are indicated in table 5.1.

Figure 5.1. DNase I footprint of the HLA-DRα 470 probe in the presence of nuclear proteins from unstimulated, and IFN-γ and IFN-γ + IFN-αβ treated colo 205, LS180 and caco 2



5.2.1. Constitutive and IFN-γ-induced protection of the HLA-DRα 470 probe

This footprint indicated the binding of complexes to the coding strand of this probe in the regions of the TAATA box, and the W, X and Y elements. This binding was not clear and was only evident from the compression of bands either side of these binding sites. The sequences of these binding sites were determined from the appropriate Maxam and Gilbert sequence.

Although the locations and nucleotide sequences of these protected regions were not easily resolved, the results from numerous such footprinting analyses indicated that binding to this promoter was consistent between the nuclear extracts of cell lines studied. This indicated that each of the cell lines studied contained similar populations of DRα promoter-binding proteins prior to treatment with stimulating cytokines. No clear differences in the binding of nuclear factors were observed after IFN-γ treatment of the cells. The protected sequences and their respective approximate nucleotide ranges within the HLA-DRα promoter are shown in **table 5.1.**

5.2.2. Protection of the HLA-DRα 470 probe by IFN-γ + IFN-αβ-treated nuclear extracts

The treatment of cells with IFN-αβ for 24hr concurrent with IFN-γ stimulation (both at 200U/ml) resulted in a novel protected region of the coding strand-labelled HLA-DRα 470 probe (**figure 5.1.**). This protection was not observed with unstimulated extracts or with those from cells treated with IFN-γ

alone. No differences in the association of nuclear factors with the HLA-DRα 470 probe were observed from IFN-αβ-treated cells when the parallel bandshift assay was performed.

The IFN-αβ-"induced" protected region was of greater size than any observed with other stimuli and, from sequence data, it spanned the nucleotides including the X box and those upstream of this towards the W box. Those sequences protected by proteins in the unstimulated and IFN-γ-stimulated nuclear extracts were also protected by factors within extracts from the IFN-γ+IFN-αβ costimulated cells. The sequence of nucleotides protected and their range in the HLA-DRα promoter is given **table 5.1.**

Table 5.1. Summary of complexes observed binding to the coding strand-labelled HLA-DRα 470 probe

	complex 1	complex 2	complex 3	IFN-αβ- "induced"
Maxam & Gilbert sequence	GGCGTAC	AGCGGTT	GTATGTGGGAC	AGACCACCCACAGGCGGTT
nucleotide range (approx.)	-103 — -97	-118 — -112	-140 — -129	-129 — -112
correspond -ing sequence in DRα	GCGTCAT	AGATGCGT	TGTGTCCTGGACCC	AAGAACCCTCCCCTAAGCAGAT
element ID	X_2 box	X box	W box	pyrimidine tract-X box

The sequences of the protein binding sites within the HLA-DRα promoter (determined by Maxam & Gilbert sequencing of the radiolabelled probe) were compared with the actual sequence of the promoter. Complexes 1 to 3 were observed in all nuclear extracts whereas the IFN-αβ-induced factor was only observed when cells had been treated concurrently with IFN-γ and IFN-αβ. Difficulties in reading the sequences were taken into consideration when comparing the experimental data with the published sequences.

5.3.Discussion

5.3.1. Technical considerations

Footprinting techniques rely on the premise that a protein factor which is associated with a specific binding site on a length of DNA will inhibit or alter the access of reagents to the bases or sugar phosphate backbone of that sequence (Goodwin, 1990). DNase I fooprinting, therefore, employs an enzymatic reaction and it is the reaction of DNase I (from bovine pancreas) upon the piece of DNA of interest which is inhibited sterically by the presence of bound proteins.

The basis of all footprinting techniques is to generate a nested series of DNA fragments from an end-labelled probe which is labelled on one strand. Where proteins have been bound and the fragmentation is inhibited, the area of protection is translated into a "gap" when the DNA fragments are separated by electrophoresis and the pattern of resolved bands is visualised. The "gap" is the footprint. Compression of bands in a footprinting "ladder" is also indicative of the presence of bound factors.

5.3.2. Practical problems and difficulties with footprint interpretation

Results from several footprinting assays performed under a variety of assay conditions were disappointing in that protected areas in footprints were not clearly defined and this led to difficult interpretation of data.

In many cases, an effect was encountered which led to low molecular weight probe fragments being lost from footprinting samples prior to their being separated by polyacrylamide gel electrophoresis. This loss of low molecular weight probe fragments was a consequence of the purification procedure after the probe had been digested by DNase. It appeared that such low molecular weight fragments were harder to release from the phenol-chloroform/aqueous phase interface in situations where relatively large concentrations of nuclear extracts had been used. It was assumed that this was the reason for the low intensity of those fragments towards the lower end of gels rather than insufficient digestion of probes.

Another complication was encountered in the reading of Maxam and Gilbert sequences in many of the assays performed; one of the reasons for this being a different, more efficient recovery system for the chemically fragmented probe sequences compared to the phenol-chloroform extraction and ethanol precipitation procedures for those probe fragments produced by enzymatic digestion. This resulted in greater, more uniform band intensity for the sequencing reactions which were revealed by autoradiography at a much faster rate than the low molecular weight footprinting probe fragments.

The relative distances of the binding sites from the labelled end of the probes employed were also cause for difficulties in the interpretation of footprinting data. Regions for the binding of nuclear factors were situated from 72 to 220 bp from the labelled end of the "coding" strand probe. This resulted in

the majority of binding sites for nuclear factors being in the least resolving part of the sequencing gel after over 5hr electrophoresis under the defined conditions. Increased lengths of time for electrophoresis were impractical and did not produce a clearer footprint.

5.3.3. Interpretation and conclusions drawn from footprinting data.

Enzymatic footprinting of the HLA-DRα 470C (coding strand) probe revealed protected regions corresponding to the X_2, X and W boxes when nuclear extracts from unstimulated and IFN-γ-stimulated colo 205 and LS180. The sequences of the protected regions given in **table 5.1.** were determined from Maxam and Gilbert sequences and, for the reasons given in **section 5.3.2.**, were not exact matches with those published. However, it was determined that the experimental sequences obtained were sufficiently consistent with published data to allow the identification of the protected sequences as those declared in **table 5.1.**

Footprinting data produced no conclusive evidence for the presence of differences in nuclear factor populations between the inducible and non-inducible colorectal tumour cell lines. Nor was there any indication that IFN-γ treatment of the cells prior to their harvesting and extraction of nuclear proteins produced a change in the existing populations of these DRα promoter-binding proteins.

These data were in contradiction somewhat to those obtained during the study of transcription factor-DNA binding interactions through the less fastidious bandshift assay analyses (see **section 4.2.**). It was concluded that the limitations of the footprinting technique employed in this study, and the relative lack of success therein, led to the revelation of no detectable differences in the binding of nuclear proteins, derived from the cell lines studied, to the part of the HLA-DRα promoter of interest here.

The results from footprinting assays may, however, be interpreted in another manner. It was possible that the patterns of binding revealed in bandshift studies were the results of protein "cofactors" binding to those transcription factors ubiquitously associated with the consensus upstream elements of the HLA-DRα promoter. Each cell line was hypothesised as having a different population of these cofactors whose association with transcription factors would not have been detectable by footprinting. The models for such hypotheses are discussed in Chapter 8.

In contrast, in the colorectal tumour cell lines colo 205 and LS180 displayed novel protection of the HLA-DRα 470C probe after treatment with IFN-αβ concurrent with IFN-γ stimulation. This "induced" factor spanned approximately 17 base pairs of the promoter (nucleotides -129 to -112) including the X box and extended to the boundary of the W box.

When the corresponding bandshift assay was performed, no such

phenomenon was observed and IFN-αβ treatment of cells did not result in a difference in the binding of nuclear proteins. It was possible that the increased footprint observed was due to the association of factors already bound altering their contact nucleotides in response to the IFN-αβ signal.

CHAPTER 6

CHAPTER 6: AN INVESTIGATION INTO THE INHIBITORY EFFECTS OF THE CYTOKINES INTERFERON-αβ AND TRANSFORMING GROWTH FACTOR-β ON IFN-γ-INDUCED REPORTER GENE ACTIVITY DIRECTED BY A 680 BASE PAIR HLA-DRα PROXIMAL PROMOTER FRAGMENT

6.1. Introduction

Reporter gene assays have been used extensively to delineate those regions of the HLA-DRα promoter which required for tissue specific and/or cytokine induced class II MHC expression *in vitro*. Such studies had shown that IFN-γ-induced and tissue-specific HLA-DRα expression required all three of the upstream consensus sequences W, X and Y - deletion or mutation of any one would obliterate the ability of cells to drive transcription from the altered promoter. However, no study has established an IFN-γ response element within this promoter either through reporter gene assays or DNA binding protein analyses.

The inhibitory effects of the cytokines interferon-αβ (IFN-αβ; type I interferon) (Morris & Tomkins, 1989) and transforming growth factor-β (TGF-β) (Czarniecki, *et* al., 1988; Epstein, *et* al., 1991 & Darley, *et al.,* 1993) on the IFN-γ-induced expression of class II MHC molecules have been reported. The effects of these inhibitory cytokines on the binding of transcription/repressor factors to regions of class II promoters and the *cis*-acting sequences or specific regions of the promoters to which they might bind have not been identified. A candidate promoter element for being the mediator of IFN-αβ action was

identified as being a consensus IFN-αβ response element, identified by sequence homology, at the distal end of the cloned 680 base pair HLA-DRα promoter fragment occupying the nucleotides -593 to -565.

In the colorectal carcinoma situation, these two cytokines were of particular importance because of the evidence for high levels of TGF-β production and secretion within tumours. In conjunction with the presence of tumour infiltrating lymphocytes, the secretion of TGF-β by tumour cells could not only down-regulate IFN-γ-induced HLA-DRα expression by the tumour cells, but also suppress the activity of the infiltrating lymphocytes whose presence might otherwise allow recognition and removal of the tumour cells.

The objectives of this section of the study into the cytokine-mediated expression of HLA-DRα were to: a) show that there was an inhibitory effect on the IFN-γ-induced expression by IFN-αβ and/or TGF-β; b) to attempt to delineate any sequence element which was responsible for such down-regulation of IFN-γ-induced expression.

6.1.1. Objectives, background and experimental considerations

The primary objective was to establish a model for the IFN-γ-induced transcription of HLA-DRα, and from this to determine the effects of IFN-αβ and TGF-β upon such induced transcription. To furnish this requirement, reporter gene assays were employed in which the 680 base pair HLA-DRα proximal

promoter fragment was used to drive the transcription of the prokaryotic gene chloramphenicol acetyl transferase (CAT).

The basis of reporter gene assays is the expression of a gene which is not present normally in the cells of interest whose transcription is modulated by a heterologous promoter - the promoter being studied. Transcription from heterologous promoters is detected by an enzymatic reaction which is indicative of the presence of the reporter gene, in this case CAT. This is a bacterial enzyme which catalyses the transfer of an acetyl group from acetyl-coenzyme A to the 3'-hydroxy position of the antibiotic chloramphenicol. The bonding of the acetyl group to chloramphenicol allows its transport into a hydrophobic phase.

This reaction system allowed the "direct scintillation" method (Eastman, 1987) for measuring the conversion of chloramphenicol to the acetate. In this method, the acetyl group of acetyl-CoA is tritiated so that its transfer to the chloramphenicol would be detected as it entered the solvent phase of a biphasic reaction consisting of aqueous reactants and scintillation fluid-containing products.

Such a system affords the means to quantitatively assess the efficiency of the heterologous promoter when the promoter and reporter genes are introduced into a cell in the form of an plasmid expression vector . Expression of the enzyme required for conversion of chloramphenicol to the solvent-phased

acetate is dependent on promoter efficiency, hence the more "active" a promoter, the greater the output of radioactive product. Thus, where the promoter structure is altered, or an environmental pressure is introduced (for example cytokine treatment of cells) such that gene expression is altered, these changes can be detected by changes in the concentration of the reporter gene product. Therefore, the supposition employed in all reporter gene assays of the type described here is the greater the radioactive output, the greater the promoter activity.

Experimental strategy

The experimental strategy for this study was designed to provide a model for the inhibitory effects of IFN-αβ and TGF-β on the IFN-γ-induced transcription of class II MHC genes in non-lymphoid cells. To enable this, the assay constituents had to satisfy certain criteria. One of the most critical stages of the assay was the transfection of the cells with the expression vector: transfectability of the cells employed and sterile plasmid preparations were essential for this. The transfection status of each "batch" of cells in an assay was assessed by their cotransfection with an expression vector for β-galactosidase, pRSVβgal (see section **2.9**). Here, the expression of the β-galactosidase gene resulted in the appearance of the functional enzyme in cytoplasmic preparations of the transfected cells, and the degree of transfection was indicated by this enzyme's ability to catalyse the chromogenic conversion of o-nitrophenyl galactoside (o-NPG) to o-nitrophenol (section **2.10.3.**). The inclusion of the β-galactosidase expression vector into the transfection reaction

provided an internal control for the transfection procedure as well as providing a means for the normalisation of assay replicates.

Another criterion which had to be met in the establishment for a model for the cytokine effects investigated was the chosen cell line's ability to be induced for the expression of class II MHC (HLA-DRα) and for this expression to be down-regulated by the actions of IFN-αβ and/or TGF-β. The cell lines of choice were those colorectal tumour cell lines which had been investigated with regard to their repertoire of HLA-DRα promoter binding proteins described in **chapters 3 to 5.** Initial experiments with these cell lines proved unsuccessful and it was decided that the technique should be established with a cell line which was known to be easily transfectible and which would show an IFN-γ-induced response when transfected with the pDRα680CAT vector. The cell line chosen was selected by its ability to furnish these requirements on the basis of the surface HLA-DRα expression determined by fluorescence-activated cell sorting (FACS) analysis (Darley, *et al.*, 1993) and its ease of handling during standard cell culture and calcium phosphate/DNA precipitate transfection.

The cell line selected for the establishment of the reporter gene assay for cytokine-regulated HLA-DRα expression was the human multiform glioblastoma cell line U138MG. In previous studies, this cell line had been shown to be class II MHC negative, yet was inducible for HLA-DRα surface expression by treatment for 24-48hr with IFN-γ. This effect had also been shown to be

abrogated by the concomitant treatment with IFN-αβ or TGF-β whereas these effects were not observed with the colorectal tumour cell lines (Darley, *et al.*, 1993). The employment of this cell line in previous reporter gene assays of this nature had proved it to be easily manipulated and transfectable with similar expression vectors (Swingler, PhD thesis, 1992).

Initial CAT assay experiments were directed to the establishment of optimal conditions for the transfection and subsequent cytokine treatment of the cell line U138MG.

6.2. Assay optimisation

6.2.1. Conditions and controls for the transfection of cells

The individual criteria responsible for successful cell growth, transfection, cytokine response and reporter gene output were determined over a series of CAT assays employing U138MG cells and the vector pDRα680CAT. The optimised protocol for the transfection of this cell line is detailed in section **2.9.** and cells were prepared for transfection by the method described therein.

Baseline CAT expression from the vector was estimated by the transfection of one set of replicates within each assay with the promoterless expression vector pCATbasic while a positive control for CAT expression was achieved by the transfection of another duplicate culture with the vector pSV2CAT where expression of the CAT gene was driven by the SV40 late

promoter. Each set of duplicate cell cultures were transfected with 20μg of the relevant CAT vector.

They were also each transfected with 10μg of the vector pRSVβgal such that the efficiencies of transfection could be assessed and the amounts of cytoplasmic extract for each duplicate employed in the final CAT enzyme assay could be normalised to compensate for differences in this transfection efficiency should they have been significant.

As described in section **2.9.**, maximal transfection efficiency was achieved when cell monolayers were transfected with calcium phosphate/DNA coprecipitates for 20-24hr at 37°C, although incubation of monolayers with precipitates for 6hr under the same conditions proved sufficient for efficient transfection.

6.2.2. Cytokine concentrations and treatment periods

Once the conditions for transfection had been optimised, the transfected cells could be treated with the cytokines of interest and the effects of such treatment assessed by the CAT assay. The following data are expressed as "fold augmentations" where stimulated CAT expression is compared to that from unstimulated controls. In the first instance, where the optimum stimulating concentration of IFN-γ was determined, the data are expressed after normalising for the number of cytoplasmic extract "units" and transfection efficiency in each assay.

6.2.2.1. Treatment of transfected cells with IFN-γ

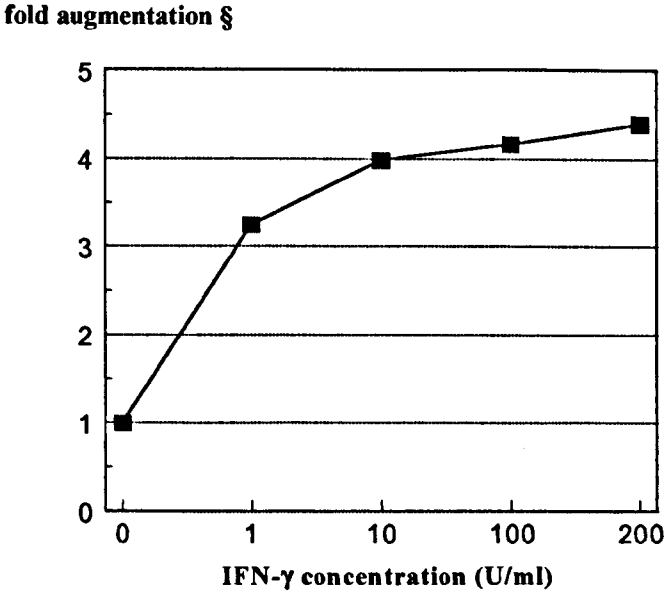
Initial experiments were designed to establish which concentration of IFN-γ, and the period of treatment, would be sufficient to yield maximal expression of CAT when activated by the HLA-DRα promoter fragment; i.e. the saturating dose of IFN-γ was established. Since the time period for maximum surface expression of HLA-D antigens had been established as being 48hr (Darley, *et al.*, 1993), this stimulation period was employed for treatment with IFN-γ here.

Although it was important to use a concentration of IFN-γ which would stimulate the cells sufficiently, it was of greater priority to establish a stimulating concentration of IFN-γ which was not saturating since this would have affected the ability of IFN-αβ and TGF-β to inhibit (if any inhibition was to be shown) the induced expression. Therefore, when the optimal concentration of stimulating IFN-γ was established, it was done so in the presence of a range of concentrations of IFN-αβ and TGF-β which showed increasing degrees of inhibition of the detected CAT expression.

Concentrations of IFN-γ from 1-200U/ml were employed to determine the saturating dose for expression of CAT directed by the HLA-DRα 680 base pair promoter fragment. **Figure 6.1.** shows the effects of the treatment of pDRα680CAT-transfected U138MG with a range of concentrations of IFN-γ. Expression of CAT was determined by the direct scintillation method as

described and is expressed as fold conversion of chloramphenicol to chloramphenicol [^{3}H]-acetate when the output from IFN-γ-treated cytoplasmic extracts were compared to those of untreated transfected cells. The results, displayed graphically in **figure 6.1.**, indicated that the stimulating IFN-γ concentration became saturating at 10U/ml due to the "plateauing" of this curve at this point.

Figure 6.1. Relative stimulation of DRα680 promoter-driven CAT reporter gene expression by increasing concentrations of IFN-γ in U138MG cells



Notes:

Results were obtained from a series of experiments in which U138MG cells were transfected for 20-24hr with a chloramphenicol acetyl transferase expression vector in which gene expression was dependent upon the heterologous HLA-DRα680 promoter fragment. After transfection, cells were treated for 48hr with the concentrations of recombinant human IFN-γ specified. Cytoplasmic extracts from transfected cells were assayed for CAT enzyme activity and expression of the CAT gene is expressed above (§) as the ratio of radioactive counts obtained from IFN-γ-treated transfected cells to those obtained form untreated cells which had been transfected with the same expression vector. This "fold" increase in expression was normalised depending on the number of extract "units" per assay.

6.2.2.2. Treatment of pDRα680CAT-transfected cells with IFN-αβ

As described in section 6.2.2.1. (above) the concentration of IFN-αβ selected to produce a significant inhibition of the IFN-γ-induced CAT expression was done so empirically and was dependent upon the stimulating concentration of IFN-γ.

Initial experiments were conducted to show inhibition of the IFN-γ-stimulated CAT expression where the cells which had been transfected with pDRα680CAT and stimulated with up to 200U/ml IFN-γ. Here "equal" concentrations, i.e. 100-200U/ml, of IFN-αβ were added to the medium above the monolayers at the same time as IFN-γ.

Although slight inhibition of CAT expression by IFN-αβ was observed under such conditions the level of inhibition was deemed not to be significant (the mean fold induction fell within 2 SEM of that observed when transfected cells were treated with IFN-γ alone). When expressed as percent augmentation (mean ± SEM) compared with that for IFN-γ stimulation alone, CAT expression was 86±9% for 10U/ml IFN-γ + 100 U/ml IFN-αβ (n=3) and 90±4% for 100 U/ml IFN-γ + 100 U/ml IFN-αβ (n=2). These results led to conclusion that although the same "unit" concentrations of IFN-γ and IFN-αβ were being employed, these were not equimolar amounts and the amount of IFN-γ would have been in a great excess of IFN-αβ in molecular terms. This was due to the assay employed to determine the "bioactive" concentration of the cytokines. The

Semliki Forest Virus assay was employed as a standard assay to determine the antiviral potency of interferon preparations. Since IFN-γ has poor antiviral properties compared to IFN-αβ, it was most likely that greater molar quantities of this cytokine were required to achieve the same degree of antiviral activity as IFN-γ in this assay system. When the two types of interferon preparations were compared out of the antiviral context, i.e. in the regulation of gene transcription as was the situation here, their potencies were more comparable and the relative amounts used could not be determined by their antiviral property-derived specific activity.

Because of the problems in determining the appropriate relative amounts of IFN-γ and IFN-αβ for the detection of the latter cytokine's inhibition of the IFN-γ-stimulated CAT expression, it was necessary to "titrate" the cytokines against each other.

The saturating dose of IFN-γ for the expression of CAT driven by the 680 base pair HLA-DRα upstream promoter fragment had been shown to be 10U/ml for 48hr. At this concentration of stimulating IFN-γ, the inhibition exhibited by the concomitant treatment with 100U/ml IFN-αβ was evident but not significant. When stimulation of CAT expression was induced by 1U/ml IFN-γ for 48hr and cells were simultaneously treated with 100U/ml IFN-αβ, the observed inhibition of transcription was greater. Here, the fold increase in CAT expression observed with 1U/ml IFN-γ- and 100U/ml IFN-αβ- treated transfected cells was

between 26% and 79% (mean±SEM=47±11%; trimmed mean±SEM=32±2.2%, n=5) of that observed with 1U/ml IFN-γ alone.

The inhibition observed by the addition of IFN-αβ to the IFN-γ-treated transfected cultures showed an inverse correlation to the degree of augmentation of CAT expression by treatment of the transfected cells with IFN-γ alone. It appeared therefore, that although a greater degree of IFN-αβ-induced inhibition was observed when stimulation of CAT expression was achieved with 1U/ml IFN-γ treatment, such a stimulating IFN-γ concentration was still saturating in a number of these assays.

Thus, the results of numerous CAT assays employing the pDRα680CAT vector in which a range of concentrations of IFN-γ were employed to induce expression of the reporter gene from this promoter showed that IFN-αβ could be employed to antagonise this induction. Although the degree of inhibition varied according to the stimulating concentration of IFN-γ, it was found that augmentation of CAT expression was reduced by the presence of 100U/ml IFN-αβ to a mean of 47% of that observed when the pDRα680CAT-transfected cells were treated with 1U/ml IFN-γ alone. The results of these experiments are shown graphically in **figure 6.2.**

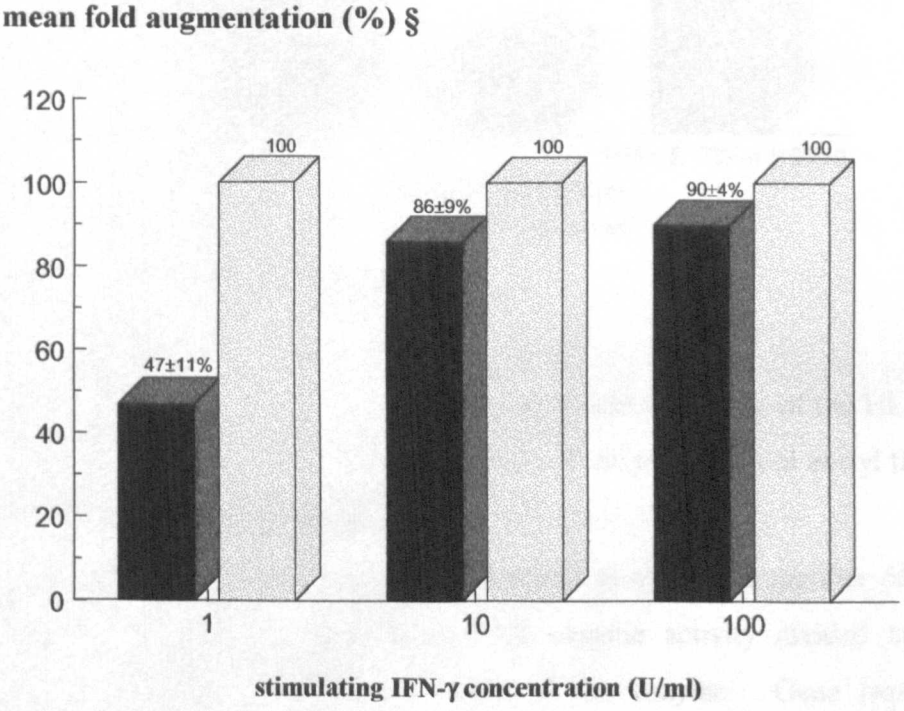
6.2.2.3. Treatment of pDRαCAT680-transfected cells with TGF-β

A similar experimental strategy was employed for the study of the effect of transforming growth factor β (TGF-β) on the expression of CAT directed by this 680 base fragment of the HLA-DRα proximal promoter. In this part of the investigation, the transfected cells were treated with a range of concentrations of IFN-γ from 1-100U/ml and treated simultaneously with 10U/ml recombinant human TGF-β_1 (British Biotechnologies) in accordance with the protocol described for the investigation of inhibition of surface expression of HLA-D antigens (Darley, *et al*; 1993). The augmentation of CAT expression was compared to that achieved by the treatment of transfected cells with IFN-γ alone.

Inhibition of the augmentation of CAT expression was observed for all stimulating IFN-γ concentrations. When 1U/ml IFN-γ was used to stimulate CAT expression and the transfected cells were treated simultaneously with 10U/ml TGF-β, the mean fold increase in CAT expression over pCATbasic was 1.94±0.33 (mean ± SEM; n=3) compared to 7.7±2.92 in the absence of TGF-β. The percentage augmentation of CAT activity ranged from 19 to 37% (27±5.2%, n=3) of that observed when transfected cells were treated with 1U/ml IFN-γ alone. Upon treatment of the transfected cells concomitantly with 10U/ml IFN-γ and 10U/ml TGF-β, the mean fold increase in CAT expression was 10.68±3.3 compared to 13.57±3.1 when the transfected cells were treated with 10U/ml IFN-γ alone. It appeared, therefore, that although inhibition of CAT expression

was observed under these conditions (percentage augmentations ranging from 70% to 86%), such inhibition was not significant. The results of these assays are portrayed graphically in **figure 6.3.**

Figure 6.2. Relative augmentation of CAT activity by IFN-γ in the presence of IFN-αβ

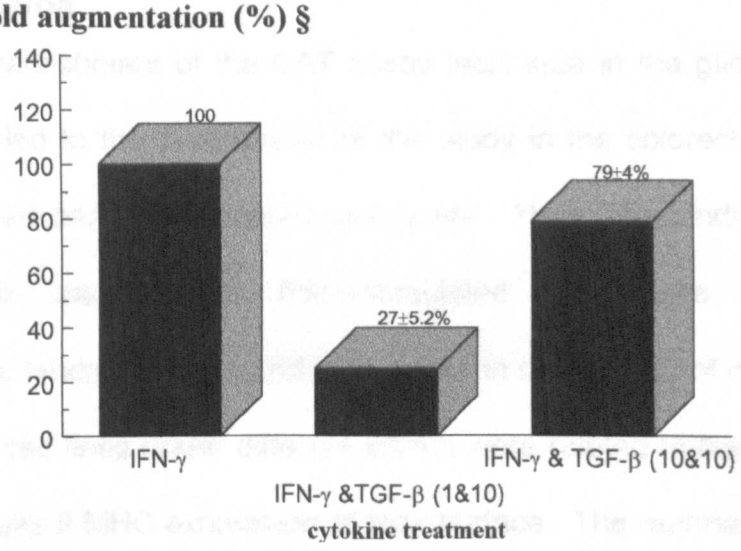


Notes:

The CAT reporter gene system was employed to assess the ability of the HLA-DRα 680 base pair promoter fragment to drive expression of chloramphenicol acetyl transferase in the glioblastoma cell line U138MG.

§: Augmentation of CAT expression is expressed as the mean number of radioactive counts obtained from cytokine-induced CAT enzyme activity divided by the mean radioactive counts due to basal expression of the enzyme from the transfected plasmid pDRα680CAT. Gene expression was augmented by 1, 10 and 100U/ml IFN-γ and was inhibited by the simultaneous treatment of pDRα680CAT-transfected cells with 100U/ml IFN-αβ. Although the absolute values for the fold increase in CAT expression were different for each stimulating IFN-γ concentration, that observed with IFN-γ alone was expressed as 100% in each case (empty). The mean (± SEM) "fold" augmentation for costimulation of cells with IFN-γ and IFN-αβ is expressed as a percentage of that observed with the appropriate IFN-γ treatment alone (filled).

Figure 6.3. Relative augmentation of CAT activity by IFN-γ in the presence of TGF-β



Notes:

The CAT reporter gene system was employed to assess the ability of the HLA-DRα 680 base pair promoter fragment to drive expression of chloramphenicol acetyl transferase in the glioblastoma cell line U138MG.

§: Augmentation of CAT expression is expressed as the mean number of radioactive counts obtained from cytokine-induced CAT enzyme activity divided by the mean radioactive counts due to basal expression of the enzyme. Gene expression was augmented by 1 or 10U/ml IFN-γ and was inhibited by the simultaneous treatment of pDRαCAT-transfected cells with 10U/ml TGF-β - concentrations of the cytokines employed in each treatment are indicated in brackets. The mean (±SEM) "fold" augmentation for each of the respective cytokine treatments is indicated as a percentage within the histogram (stimulation with IFN-γ alone is shown as 100% regardless of concentration).

6.3. Reporter gene assays with pDRα680CAT in colorectal tumour cell lines

6.3.1. Introduction

The establishment of the CAT assay technique in the glioblastoma cell line U138MG led to the progression of the study in the colorectal tumour cell lines which had been investigated previously. Here, the binding of nuclear proteins from untreated or IFN-γ-stimulated cells were employed in electrophoretic binding assays and differences in the binding of nuclear factors from different cell lines under different stimuli were related to their ability to be induced for class II MHC expression at their surface. The reporter gene assays described below were devised to relate such transcription factor binding to transcriptional function of the HLA-DRα promoter in the human colorectal cell lines studied.

6.3.1.1. Effects of interferon-γ treatment upon cell surface expression of class II MHC in human colorectal tumour cell lines

The colorectal tumour cell lines investigated in this study had been selected from a larger panel of candidate cell lines because of differences in their abilities to respond to IFN-γ with respect to surface expression of MHC class II antigens. The use of fluorescence activated cell sorting (FACS) techniques in which the surfaces of cells were stained with specific fluorescent antibodies to HLA-D allowed this identification (Darley, *et al,* (1993)). Of the colorectal cell lines already studied two, LS180 and caco 2, had been selected because of their inability to express class II MHC antigens after treatment with

IFN-γ, while colo 205 had been used for the converse reason. None of these cell lines expressed class II MHC antigens constitutively. Due to its relative ease of handling and its inducibility for class II MHC, a fourth colorectal cell line, HT29, was introduced for this investigation. In the investigation described by Darley, *et al,* (1993), this cell line was also shown to display no constitutive class II MHC surface antigens, but were strongly induced by treatment with IFN-γ. In contrast to colo 205, however, HT29 is a cell line which grows as a monolayer and this proved desirable for the type of experimentation undertaken here.

6.3.1.2. The effects of IFN-αβ and TGF-β on IFN-γ-induced expression of class II MHC antigens by colorectal tumour cell lines.

The analysis of the colorectal cell lines employed in this study as regards their expression of class II MHC antigens at the cell surface in response to IFN-γ also established their respective responses to TGF-β. Darley, *et al,* (1993) described the growth-inhibitory and MHC class II down-regulatory properties of TGF-β in a panel of cell lines and these data were of use when the cell lines to be employed in this investigation were chosen. It was shown that none of the colorectal cell lines displayed TGF-β-related inhibition of IFN-γ-induced MHC class II antigen expression and colo 205 and LS180 were growth-inhibited by TGFβ while HT29 showed no response in this regard. On the other hand, U138MG, the glioblastoma cell line employed in initial parts of reporter gene studies, had its growth stimulated by treatment with TGF-β and

IFN-γ-induced surface expression of class II MHC antigens was also down-regulated. Hence, it was not expected that reporter gene assays employing pDRα680CAT-transfected colorectal tumour cells would show any response to TGF-β treatment and only colo 205 and HT29 were expected to display induction of IFN-γ-induced CAT expression.

No data for the inhibitory effects of IFN-αβ on IFN-γ-induced HLA-DR antigen expression were provided by the study mentioned above, however, this cytokine's inhibitory properties upon the expression of class II MHC expression have been well documented (Morris, and Tomkins, 1989; Ling, *et al.*, 1985). Since IFN-αβ had already been shown to antagonise the IFN-γ-induced expression of pDRα680CAT in U138MG, it was assumed that the same methodology could reveal any similar effects, should they occur, in the colorectal cell lines chosen. The same reasoning was true for the potential effects of TGF-β treatment of cells concurrent with IFN-γ stimulation.

6.3.1.3. Other considerations with the employment of non-inducible cell lines

The inability of cells to be induced for class II MHC expression has been the subject of many studies into the transcriptional aspects of regulation of class II genes. A number of these studies, particularly in the case of class II-deficient combined immunodeficiency (CID) have concentrated on the accessibility of class II promoters to transcription factors and have postulated that cell types which should be able to be induced by IFN-γ for class II MHC expression are

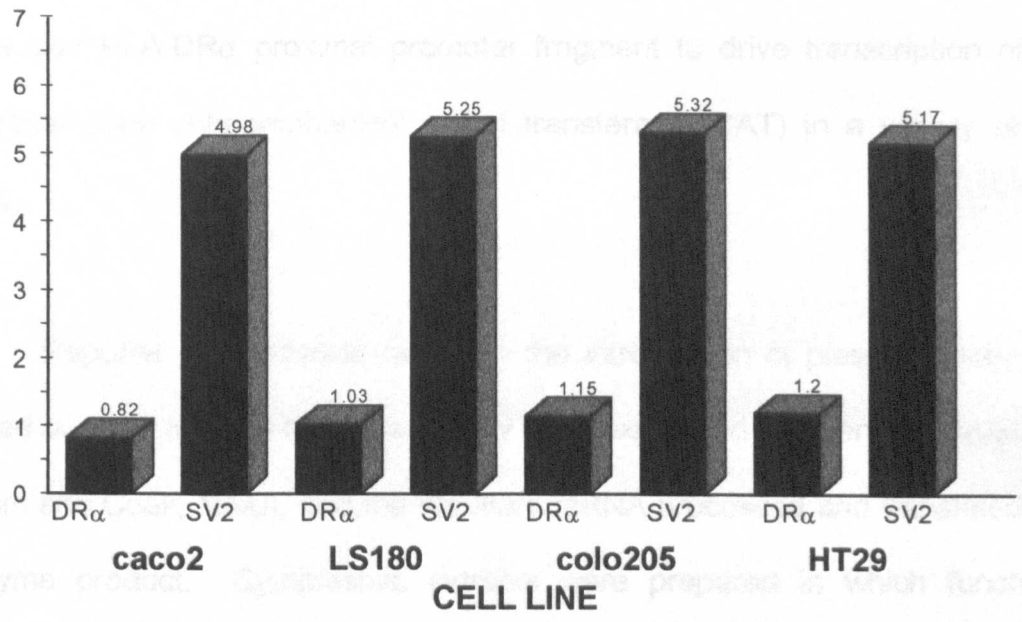
unable to because of this. This "inaccessibility" of promoters within this MHC locus may be due to promoter configuration (perhaps due to chromatin structure) and is secondary to a defective class II promoter factor whose rôle it is to organise these promoters into an accessible configuration (Kara and Glimcher, 1993). In the situation of some of the colorectal tumour cell lines studied here, it was possible that class II uninducibility was a result of a similar defect in promoter accessibility rather than a deficiency in a transcription factor responsible for IFN-γ-induced HLA-DRα expression. Because of this, it was a possibility that a deficiency in a class II promoter organising factor would be indicated by IFN-γ-induced expression of CAT directed by the "naked" HLA-DRα promoter fragment in this *in vitro* system.

Hence, the investigation here would provide information regarding the uninducibility of the cell lines caco 2 and LS180 to express class II MHC in response to IFN-γ stimulation as well as determining whether TGF-β and IFN-αβ could inhibit the IFN-γ-induced expression of CAT in the inducible cell lines colo205 and HT29.

6.3.2. Interferon-γ-induced expression of chloramphenicol acetyl transferase by pDRα680CAT-transfected colorectal tumour cell lines

A number of CAT assays were performed with each of the colorectal tumour cell lines in an attempt to establish whether there would be a response to IFN-γ in the context of the reporter gene assay. The results of these are summarised in **figure 6.4.** As indicated by this graphic, the effects of IFN-γ treatment on pDRα680CAT-transfected colorectal tumour cells were minimal compared to those results observed with U138MG although a slight, yet insignificant increase in CAT expression by the inducible cell lines colo 205 and HT29. The lack of IFN-γ-induction of CAT expression of all colorectal cell lines was irrespective of transfection efficiency, as assessed directly by β-galactosidase assay from the transfected vector pRSVβ-gal and indirectly by CAT expression by pSV2CAT-transfected cells. Although the transfection efficiency of these cells was not as great as that obtained with U138MG, ranging from 20 to 50% of the transfection levels obtained with the glioblastoma cell line, CAT expression due to pSV2CAT was seen to be at the same level as observed in U138MG. These results are incorporated into **figure 6.4.**

Figure 6.4. The response of pDRα680CAT-transfected colorectal tumour cell lines to IFN-γ



Notes:

The human colorectal tumour cell lines LS180, caco 2, colo 205 and HT29 were employed in a series of CAT assays in which each was transfected with the expression vector pDRα680CAT or pSV2CAT. Presence of chloramphenicol acetyl transferase in cytoplasmic extracts was dependent upon promoter activity: "augmentation" of pDRα680CAT was attempted with up to 100U/ml IFN-γ while expression of CAT driven by the SV40 late promoter was constitutively high and acted as a positive control. In the histogram, "DRα" refers to cells which had been transfected with pDRα680CAT and treated with 100U/ml IFN-γ for 48hr prior to preparation of cytoplasmic extracts and chloramphenicol acetyl transferase assay; "SV2" refers to cells (within the same assay) which had been transfected with pSV2CAT and assayed for CAT enzyme activity without cytokine treatment. "Fold augmentation" was measured by comparing the radioactive counts from IFN-γ-treated pDRα680CAT transfected cells with those which remained untreated, whereas that for pSV2CAT-transfected cells was measured by comparison of counts with those cells transfected with the comparable pOCAT vector.

6.4. Discussion

Reporter gene assays were employed to assess the ability of the 680 base pair HLA-DRα proximal promoter fragment to drive transcription of the bacterial gene chloramphenicol acetyl transferase (CAT) in a variety of cell lines.

Reporter gene studies relied on the introduction of plasmid constructs, based on CAT, into the cells where they were expressed transiently in episomes (Alam and Cook, 1990), and the resultant mRNA processed and translated into enzyme product. Cytoplasmic extracts were prepared in which functional enzyme product was determine. Thus, reporter gene assays do not provide a direct assessment of transcription from the heterologous promoter, rather a measurement of gene expression. Although the level of enzyme activity is likely to be related to steady-state mRNA levels and frequency of transcription initiation at the promoter, RNA stability post-transcription should be taken into account. The same is also true for translational efficiency where although the transcription was efficient, translation into the functional enzyme product did not occur. These considerations were taken into account when interpreting data obtained from CAT assays.

6.4.1. The U138MG human glioblastoma cell line displayed IFN-γ-induced CAT expression driven by the 680 base pair HLA-DRα promoter fragment

6.4.1.1. Summary of results

The results from the CAT assays employing the multiform glioblastoma cell line U138MG provided a model for rôles of the cytokines IFN-γ, IFN-αβ and TGF-β in the control of the transcription of class II MHC genes. Over a series of assays it was shown that IFN-γ could induce the expression of the enzyme chloramphenicol acetyl transferase when the reporter gene was cloned downstream of the 680 base pair HLA-DRα proximal promoter fragment in the expression vector pDRα680CAT. This induced transcription, defined by the activity of the CAT enzyme product and normalised for transfection efficiencies and units of cytoplasmic extract employed per assay, ranged from 7.7 to 15.5 fold the basal transcription of the gene when transfected cells were treated with between 1 and 200U/ml IFN-γ for 48hr (see **figure 6.1.**).

The stimulating concentration of IFN-γ at which augmentation of CAT expression was maximal was found to be 10U/ml. At this stimulating concentration of IFN-γ, the antagonistic effect of IFN-αβ treatment upon CAT expression was not significant and augmentation of CAT activity was 85% of that observed with IFN-γ alone. However, when stimulation of CAT expression by transfected U138MG was augmented by 1U/ml IFN-γ for 48hr and the cells were also treated with 100U/ml IFN-αβ, the induction of CAT expression was reduced to 47%, on average, of that displayed by cells stimulated with IFN-γ

alone (see section 6.2.2.2, and **figure 6.2.**).

When TGF-β was used in an attempt to antagonise the IFN-γ-induced augmentation of CAT expression, strong inhibition was observed with 10U/ml TGF-β when 1U/ml IFN-γ was used as the stimulating concentration. Under these conditions the augmentation of CAT expression was reduced to 27% of that observed with IFN-γ alone (see section 6.2.2.3.). When the stimulating concentration of IFN-γ was increased to 10U/ml and TGF-β was also employed at 10U/ml, the augmentation of CAT expression was 78% of the maximum, indicating that this IFN-γ concentration was saturating. The combined results of these experiments are shown in **table 6.1.**

Table 6.1. Effects of cytokines on chloramphenicol acetyl transferase augmentation by U138MG transfected with pDRα680CAT

stimulating IFN-γ concentration*	Percentage Augmentation** (mean±SD)	
	IFN-αβ, 100U/ml	TGF-β, 10U/ml
1U/ml	47±28% (32±8%)†	27±9%
10U/ml	86%	78±8%
100U/ml	90%	ND

Results were obtained from U138MG cells following transfection with the CAT expression vector pDRα680CAT and stimulation with the concentrations of the cytokines specified. **Percentage augmentation refers to the mean fold increase in radioactive counts due to chloramphenicol acetyl transferase activity expressed as a percentage of that which was observed with transfected cells treated with IFN-γ alone. *Stimulating IFN-γ concentration refers to the concentration of IFN-γ employed to induce CAT activity in pDRα680CAT-transfected cells. In all cases, cytokine treatment was for 48hr prior preparation of cytoplasmic extracts and CAT assay. † is the trimmed mean for this data set. N.D. = not determined

6.4.1.2. Conclusions drawn from CAT assays with U138MG and pDRα680CAT

Thus, the results obtained from reporter gene assays employing pDRα680CAT-transfected U138MG glioblastoma cells provided a model for the inhibition of IFN-γ-induced HLA-DRα expression by IFN-αβ and TGF-β. It was shown that this inhibition was possible with a 680 base pair HLA-DRα promoter fragment which spanned the nucleotides -640 to +40, relative to the start of transcription. The assumptions made with reporter gene assays led to the assumption that observed inhibition was transcriptional. This has been

supported by other studies with primary astrocytes which have shown that TGF-β exhibits an inhibitory effect upon the IFN-γ induction of class II MHC mRNA and surface protein as well as affecting promoter activity (Panek, *et al.*, 1995).

Other evidence in support of the observations encountered here was provided in independent studies, both employing the CAT assay reporter gene system, in Reimold, *et al.*, (1993) and Devajyothi *et al.*, (1993). The former investigation studied the inhibition of IFN-γ-induced CAT activity by TGF-β in a melanoma cell line (Hs294T9(c)) and concluded that this inhibition was exerted through the W-X_1 region of the promoter although there was no evidence for the interaction of TGF-β-induced transcription factors within this region. Devajyothi *et al.*, (1993) employed CAT reporter gene assays in the astrocytoma cell line CRT in which a nested series of 5' deletion mutants of the HLA-DRα promoter from -2.2 kilo bases to -267 bases was cloned into CAT expression vectors. Again, it was shown that 267 base pairs of upstream sequence was required for IFN-αβ and TGF-β to elicit their inhibitory effects upon IFN-γ-induced CAT activity in this cell line. This study was of particular interest since it was performed in a cell line of neural origin and the data obtained were considered to reflect somewhat those obtained here with U138MG. The astrocytoma cells displayed similar levels of IFN-γ-induced augmentation of CAT expression to U138MG and the inhibition, expressed as a percentage, was 76% for IFN-αβ and 60% for TGF-β. These levels of inhibition were greater than those seen in the present investigation, yet different concentrations and preparations of the

respective cytokines were employed. Hence, a qualitative parallel was observed between these two related cell lines.

6.4.2. Reporter gene assays in human colorectal tumour cell lines

Reporter gene assays employing the four human colorectal tumour cell lines and the vector pDRα680CAT proved to be "unsuccessful" in increasing the understanding of cytokine-induced HLA-DRα promoter-driven reporter gene expression which had been gained by the use of U138MG. Although a number of assays were performed for each of the cell lines, none displayed any IFN-γ-induced CAT activity when transfected with the pDRα680CAT vector and treated with a range of IFN-γ concentrations for 48hr. This was not unexpected for the non-inducible cell lines caco 2 and LS180, yet was completely unexpected for the "inducible" colo 205 and HT29 colorectal tumour cell lines.

The potential for these data to have been erroneous were addressed by repeated experimentation with several preparations of solutions employed during transfection and CAT assay as well as with different batches of plasmids.

6.4.2.1. Transfection efficiencies of colorectal tumour cell lines

It could have been argued that the human colorectal tumour cell lines employed did not show any CAT activity from the plasmid pDRα680CAT because they were not transfected efficiently, however evidence obtained from controls showed that this was not the case here. Internal controls incorporated into each assay indicated that, although transfection rates were decreased compared to those observed with U138MG, the cells had been successfully transfected with pRSVβ-gal. The assumption adhered to when employing such cotransfection protocols was that β-galactosidase activity in cytoplasmic extracts of cells which had also been transfected with another plasmid (in this case, pDRα680CAT) would reflect the "transfection status" of those cells for all the vectors involved. That is to say, the greater the β-galactosidase activity, the greater the transfection efficiency for that event. It was therefore assumed that the cell lines employed here had been transfected efficiently with all plasmids when the β-galactosidase "signal" was seen to be significant.

Another piece of evidence to support the efficient transfection of the cells was the constitutively high expression of chloramphenicol acetyl transferase due to transfection with pSV2CAT. This was also significant in that it showed that all of these cell lines had the ability to process CAT transcripts efficiently and produce the final enzyme product. Thus, the apparent lack of induction of CAT from the pDRα680CAT vector was unlikely to be due a defect in the cell lines' transcription/translation machinery with regards to this gene product.

One other possibility for these cell lines' inability to show induction of CAT expression from the pDRα680CAT vector was that the vector itself was damaged or that a poor plasmid preparation was employed for these particular assays. Each plasmid preparation was assessed for purity by spectrophotometry at 260 and 280nm and their integrities were assessed by electrophoresis through 1% agarose (see section **2.8.3.**). Also, in many of the assays employing colorectal tumour cell lines, assays employing U138MG cells, in which CAT activity from this plasmid was augmented as described, were performed simultaneously.

Hence, it was assumed that since transfection was efficient, the colorectal cell lines were able express CAT from the SV40 late promoter and U138MG were inducible for DRα680-driven CAT expression when transfected simultaneously, the colorectal tumour cell lines employed here were unable to exhibit IFN-γ-induced CAT expression from pDRα680CAT for reason/s other than those experimental considerations addressed above.

Although not tested experimentally, the inability of the human colorectal tumour cell lines to be induced for HLA-DRα680 CAT expression by IFN-γ was most probably explained by the promoter fragment itself. That is to say that the 680 base pair promoter fragment employed in these assays either contained a tissue-specific repressor or lacked a tissue-specific enhancer element. Although no published data were available to support this conclusion the presence of the octamer element within the HLA-DRα promoter, involved in

B-cell-specific expression, demonstrates that tissue specific enhancer elements do have a rôle in the control of this gene's expression.

Because of their inability to display IFN-γ-induced CAT expression when transfected with pDRα680CAT, the colorectal tumour cell lines could not be assessed for the effects of IFN-αβ and TGF-β treatment in this regard.

CHAPTER 7

CHAPTER 7: AN INVESTIGATION INTO THE INHIBITORY EFFECTS OF THE CYTOKINES INTERFERON-αβ AND TRANSFORMING GROWTH FACTOR-β ON IFN-γ-INDUCED REPORTER GENE ACTIVITY DIRECTED BY A 320 BASE PAIR HLA-DRα PROXIMAL PROMOTER FRAGMENT

7.1. Introduction

As discussed previously, reporter gene assays have been used to demonstrate the importance of the consensus upstream sequences in the control of HLA-DRα expression in a variety of cell types. **Chapter 6** demonstrated the ability of a 680 base pair proximal promoter fragment to mediate the augmentation of CAT expression in human glioblastoma cells (U138MG) transfected with a CAT plasmid whose expression was driven by this heterologous promoter after the cells' treatment with IFN-γ.

This HLA-DRα promoter fragment was also shown to have the ability to mediate the IFN-αβ and TGF-β suppression of IFN-γ-induced CAT expression in this cell line. Although no consensus sequence for the binding of a TGF-β-induced "repressor" factor was present in the HLA-DRα promoter, a consensus IFN-αβ response element had been identified in the region of the promoter between the nucleotides -593 to -565 relative to the start of transcription.

The following chapter describes how a 5' deletion of the DRα680 fragment was employed to determine whether this sequence was alone responsible for the antagonistic effects of IFN-αβ treatment upon the IFN-γ-induced expression of chloramphenicol acetyl transferase in the appropriately transfected cells.

7.1.1. The pDRα320CAT expression vector

This reporter gene vector was prepared by the restriction endonuclease digestion of the plasmids pCAT-basic and pBS(KS)$^+$DRα680 5'→3' with Xba I. The resultant DRα320 fragment and the recipient pCAT-basic plasmid were ligated as described in **chapter 2**. The 5' deletion of the 680 base pair HLA-DRα promoter fragment resulted in the excision of the putative IFN-αβ regulatory element. Transfection of the appropriate cell line with this vector and subsequent treatment with IFN-γ in the presence or absence of IFN-αβ was expected to reveal whether the deleted consensus IFN-αβ element was responsible for the IFN-αβ-mediated suppression of IFN-γ-induced CAT expression observed with the vector pDRα680CAT in U138MG cells.

7.1.2. Cell lines and experimental strategy

The cell lines of choice were those derived from human colorectal tumours such that the binding of nuclear proteins to the HLA-DRα promoter and to individual sequence elements could be related to transcriptional function. For the reasons given in **chapter 6** regarding the relative technical difficulties in

handling these cell lines, it was again decided to optimise the assay in a "proven" cell line.

The human glioblastoma cell line U138MG was again used to establish the optimal conditions for the study cytokine regulation of HLA-DRα expression in reporter gene assays. This cell line had proved to be easy to manipulate and was readily transfectable with plasmid vectors.

The investigation to delineate the regions of the HLA-DRα promoter responsible for the observed IFN-αβ and TGF-β suppression of IFN-γ-induced expression was continued with this cell line in further experiments employing the deleted HLA-DRα promoter fragment-containing vector pDRα320CAT. Here the strategy for experimentation was the same as that described for the pDRα680CAT vector. Initial experiments determined the response of pDRα320CAT-transfected U138MG to varying concentrations of IFN-γ such that the saturating dose could be determined. Following this titration of IFN-γ and optimisation of transfection, incubation and assay conditions, it was possible to assess the transfected cells' response to IFN-αβ and TGF-β.

The study was then continued in the human colorectal tumour cell lines colo 205, HT29, LS180 and caco 2. The responsiveness of these cell lines to the cytokines IFN-γ, IFN-αβ and TGF-β as regards surface expression of class II MHC antigens has been described previously (summarised in **table 2.1.**).

This investigation was undertaken despite the apparent uninducibility of these cell lines for CAT expression from the vector pDRα680CAT. It was of interest to confirm the results obtained here since such data would have indicated a general inability of these cell lines to express this type of reporter gene vector. The continued inability to express CAT when driven by the truncated promoter would have been indicative that a tissue-specific enhancer element was absent. However, had pDR320CAT-transfected colorectal tumour cell lines expressed CAT, it would be assumed that removal of the 360 base pair upstream promoter sequence released the cells from a tissue-specific repressor.

Again, the control vectors pRSVβ-gal and pSV2CAT were employed to assess these cell lines' ability to be transfected and to transcribe and process CAT mRNA.

7.2. Reporter gene assays with U138MG and pDRα320CAT

7.2.1. Conditions for cell culture and transfection

The cell line U138MG was cultured *in vitro* in complete DMEM under the conditions described in materials and methods (**chapter 2**). Passage of the cultures was performed such that weekly preparations of confluent cells were available for employment in CAT assays as described in section **2.9.1.** Calcium phosphate-mediated transfection of cell lines with expression and control vectors was performed as described previously.

7.2.2. Augmentation of DRα320-mediated CAT expression by IFN-γ

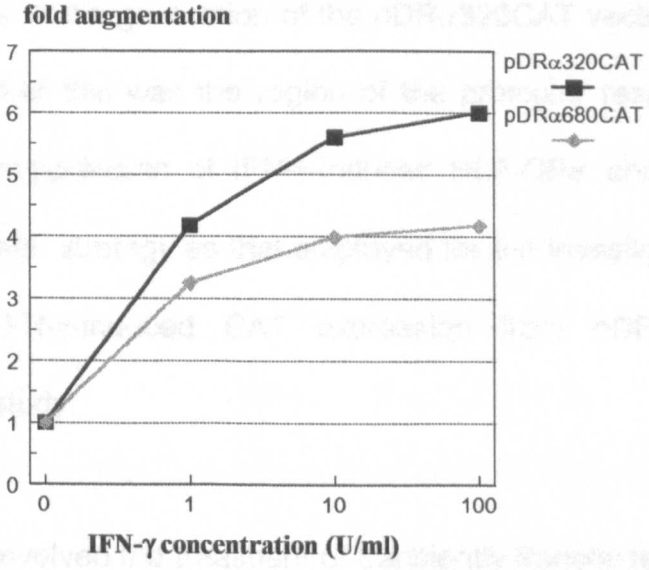
The same strategy for the IFN-γ-stimulation of pDRα320CAT-transfected U138MG was employed as that described for those cells transfected with pDRα680CAT. Cells were stimulated with a range of IFN-γ concentrations from 1 to 100U/ml for 48hr post glycerol shock. The response of pDRα320CAT-transfected U138MG to IFN-γ ranged from 4.2 to 6 fold the CAT expression seen with untreated transfected cells. These responses were normalised for the number of units of cytoplasmic extracts employed within each assay. **Figure 7.1.** is a graphical representation of the IFN-γ-induced CAT expression by pDRα320CAT-transfected U138MG cells.

7.2.2.1 Comparison of IFN-γ responses with pDRα320CAT and pDRα680CAT

On average, the IFN-γ-stimulation of pDRα320CAT-transfected U138MG cells yielded a greater expression of CAT than the same treatment of those cells transiently transfected with pDRα680CAT. This was based on the comparison of the mean fold increase in CAT expression per unit of cytoplasmic extract for each of the concentrations of IFN-γ stimulation. Although the results did not appear to be significantly different (the respective mean values fell within 2 SEM of each other), a trend was observed for all of the IFN-γ stimulations. For example, the mean fold increase in CAT expression stimulated by 1U/ml IFN-γ was 4.18±1.44 (mean±SEM) for pDRα320CAT (n=3) compared to 3.25±0.83 for pDRα680CAT (n=4).

Although these observations were not statistically proven, it appeared that removal of the upstream-most 360 base pairs of the promoter released some repression which might have been imposed by this region. This gave a primary indication that this upstream region of the promoter did indeed contain a sequence or sequences involved in the down-regulation of HLA-DRα expression in this cell line. This comparison between the CAT expression observed for the two vectors is shown graphically in **figure 7.1.**

Figure 7.1. Augmentation of CAT expression by IFN-γ in pDRα320CAT-transfected U138MG cells



Notes:

CAT assays were employed to assess the IFN-γ-mediated gene expression through the HLA-DRα 320 base pair promoter fragment in the U138MG human glioblastoma cell line. Cells were transfected transiently with the vector pDRα320CAT then treated for 48hr with varying concentrations of IFN-γ. Gene expression was measured as a function of CAT enzyme activity in the cytoplasmic extracts of transfected cells. Fold augmentation was calculated by dividing those radioactive counts obtained from IFN-γ-induced CAT enzyme activity by those obtained untreated transfected cells this was then normalised to take into account the number of "units" of cytoplasmic extract used. Fold augmentations for individual IFN-γ stimulating concentrations were also normalised for transfection efficiency from the expression of β-galactosidase.

The comparable data for the IFN-γ-stimulated augmentation of CAT expression from U138MG transfected with pDRα680CAT are also shown.

7.2.3. Suppression of IFN-γ-induced CAT expression by IFN-αβ

The removal of the putative IFN-αβ consensus element from the HLA-DRα promoter in the generation of the pDRα320CAT vector was intended to determine whether this was the region of the promoter responsible for the IFN-αβ-mediated suppression of IFN-γ-induced HLA-DRα and pDRα680CAT expression. A similar strategy as that employed for the investigation of IFN-αβ suppression of IFN-γ-induced CAT expression from pDRα680CAT was employed for this study.

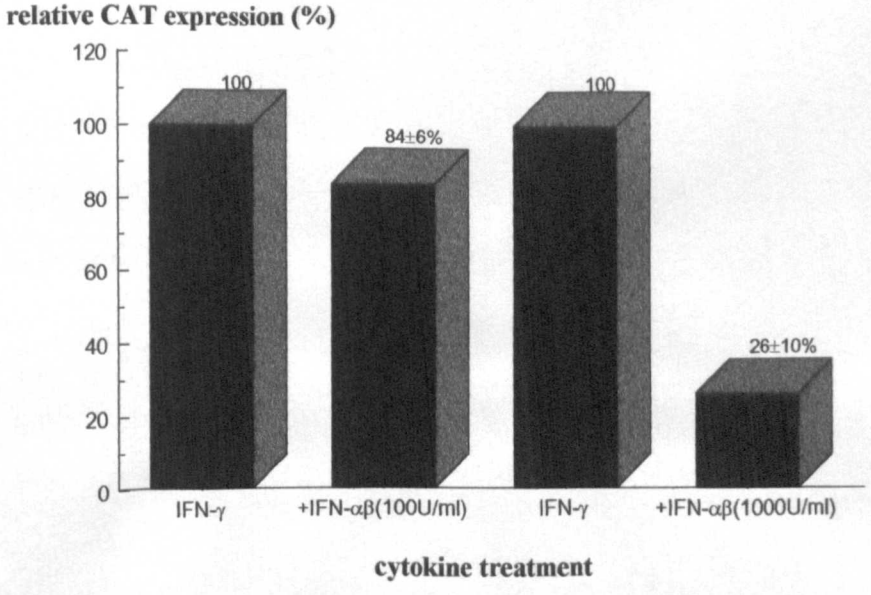
This study involved the treatment of transiently transfected U138MG with IFN-γ concurrent with IFN-αβ; results from the study with cells transfected with pDRα680CAT were utilised to determine initial conditions for this treatment of the cells with cytokines. The cytokine treatment of U138MG which had been transfected with pDRα320CAT was performed with 1U/ml IFN-γ concurrent with 100U/ml IFN-αβ for 48hr post glycerol shock. This treatment achieved a level of CAT expression whose mean was 84±6% (mean±SEM; n=3) of that observed with 1U/ml IFN-γ alone.

It appeared, therefore, that the deletion of the HLA-DRα promoter in the construction of this expression vector had resulted in the reduced potency of IFN-αβ in the suppression of IFN-γ-induced CAT expression. The same cytokine treatment of pDRα680CAT transfected U138MG cells resulted CAT expression being reduced to a mean of 47% of that observed with IFN-γ

treatment alone (see section 6.2.2.2.).

When the pDRα320CAT-transfected cells were stimulated with 1U/ml IFN-γ concurrent with 1000U/ml IFN-αβ, CAT enzyme activity was reduced to 26±10% (mean±SEM; n=3) of that observed in cells stimulated with IFN-γ alone. This result indicated that the removal of the putative IFN-αβ consensus element was not sufficient to render the promoter completely unresponsive to the inhibitory effects of this cytokine. The responses of pDRα320CAT-transfected U138MG to IFN-γ and IFN-αβ are represented graphically in **figure 7.2.**

Figure 7.2. Relative suppression of IFN-γ-induced CAT expression by IFN-αβ

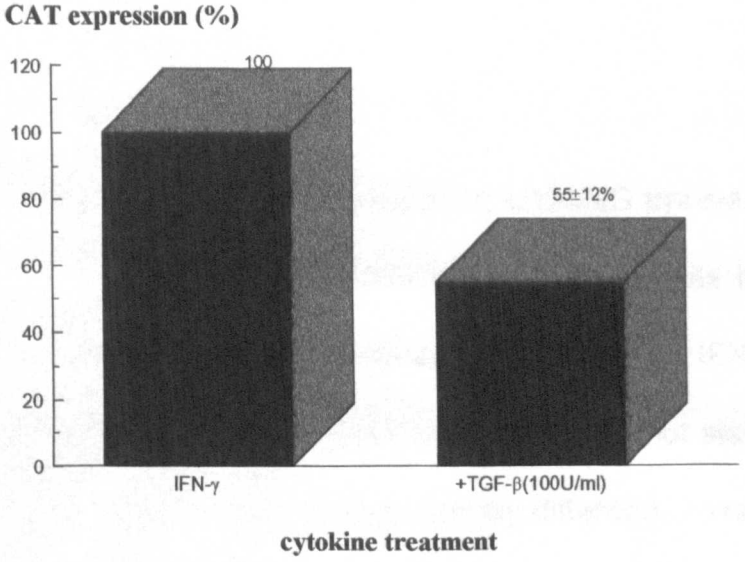


Notes:
U138MG cells were transiently transfected with pDRα320CAT and treated with 1U/ml IFN-γ alone or with IFN-γ (1U/ml) concurrent with IFN-αβ at 100 or 1000U/ml. CAT expression is represented as a percentage where the augmentation observed with IFN-γ alone is given as 100% and the CAT expression observed when cells were treated concurrently with IFN-αβ is expressed as a percentage of this.

7.2.4. Suppression of IFN-γ-induced CAT expression by TGF-β

As with the investigation into IFN-αβ suppression of IFN-γ-induced CAT expression in pDRα320CAT-transfected U138MG, the same strategy for study of TGF-β-mediated inhibition of expression was employed as with pDRα680CAT transfected cells. The cytokine concentrations employed for the stimulation of CAT expression with IFN-γ and the consequent inhibition by TGF-β were 1U/ml and 100U/ml, respectively. Such treatment with TGF-β resulted in an average augmentation of CAT expression of 55±12% (mean±SEM) of that observed with IFN-γ alone. This level of CAT expression was approximately double that observed when pDRα680CAT-transfected cells, where CAT expression was observed at 27±9% (mean±SEM) of the IFN-γ-induced controls (see section 6.2.2.3). The results for this suppression of IFN-γ-induced CAT expression by TGF-β are represented graphically in **figure 7.3.**

Figure 7.3. Relative inhibition of IFN-γ-induced CAT expression by TGF-β



Notes:

U138MG cells were transiently transfected with pDRα320CAT and treated with 1U/ml IFN-γ alone or with IFN-γ (1U/ml) concurrent with 100U/ml TGF-β. CAT expression is represented as a percentage where the augmentation observed with IFN-γ alone is given as 100% and the CAT expression observed when cells were treated concurrently with TGF-β is expressed as a percentage of this.

7.2.5. Comparison of CAT expression in U138MG transfected with pDRα320CAT and pDRα680CAT

The human glioblastoma cell line U138MG was employed to assess the function of the upstream 340 base pair region of the HLA-DRα promoter with respect to IFN-αβ and TGF-β-mediated suppression of IFN-γ-induced CAT expression. This region contained a putative IFN-αβ response element and its deletion in the construction of pDRα320CAT was intended to verify or discount this element's involvement in IFN-αβ-mediated down-regulation of IFN-γ-induced expression from the HLA-DRα promoter. Deletion of this portion of the upstream promoter was also intended to determine whether any distal

elements were responsible for cytokine-mediated or constitutive repression of expression.

Interferon-γ-induced CAT expression in U138MG transiently transfected with pDRα320CAT tended to be greater than that from cells transfected with pDRα680CAT and treated with the same concentration of IFN-γ (see section 8.2.2.1). Although this increase in CAT expression was not statistically proven (the increase in expression was not significantly different), it was a trend which was observed for all concentrations of IFN-γ stimulation. These results indicated that removal of the 360 base pair upstream region of the HLA-DRα promoter also released an inherent repressive *cis*-acting element which acted within the upstream part of the promoter.

Concurrent treatment of pDRα320CAT-transfected U138MG cells with IFN-γ and IFN-αβ at the concentrations of 1U/ml and 100U/ml, respectively was insufficient to show the inhibition of CAT expression observed previously for these cytokine concentrations in pDRα680CAT-transfected cells. Under these conditions, CAT expression was observed at a mean of 84% of that observed when pDRα320CAT-transfected cells were treated with 1U/ml IFN-γ alone, compared to 47% in cells transfected with pDRα680CAT. It appeared, therefore, that the putative IFN-αβ response element was effective in this cytokine's involvement in the suppression of gene expression from the HLA-DRα promoter. The suppression could be reinstated by increasing the

concentration of IFN-αβ to 1000U/ml and under these conditions the CAT expression was observed to be 26% of that with 1U/ml IFN-γ alone. Although, the upstream promoter region appeared to be involved in the IFN-αβ-mediated suppression of IFN-γ-induced gene expression, this suppression was not solely mediated through this region. Whether the removal of suppression was due to a specific IFN-αβ response element or whether removal of a more general *cis*-acting repressor element was responsible could not be determined, however, the trend towards increased IFN-γ-induced CAT expression from the 320 base pair promoter fragment indicated that the latter may be true.

Treatment of pDRα320CAT-transfected U138MG with 1U/ml IFN-γ concurrent with 100U/ml TGF-β resulted in CAT expression being 55% of that observed when cells were treated with 1U/ml IFN-γ alone. As with the other cytokine treatments of cells transfected with this vector, this level of CAT expression was greater than when the same cell line was transfected with pDRα680CAT and treated with the same cytokines (here the CAT expression was 27% of maximal). Again, levels of CAT expression were greater with pDRα320CAT-transfected cells than with cells transiently transfected with pDRα680CAT. This indicated that a general repressor was present in the 360 base pair fragment of the HLA-DRα promoter deleted in the construction of pDRα320CAT.

In conclusion, deletion of the 360 base pair upstream promoter region from pDRα680CAT in the construction of pDRα320CAT resulted in a CAT expression vector which was more effective in expression of the reporter gene CAT after induction of transiently transfected cells with IFN-γ. Although the suppression of CAT expression with 100U/ml IFN-αβ appeared to be abolished - CAT expression was restored to near maximal - when the concentration of IFN-αβ was increased to 1000U/ml, the inhibition was restored. Suppression of CAT expression by TGF-β was also reduced with this plasmid. It appeared, therefore, that removal of the upstream region of the HLA-DRα promoter caused the removal of a general repressor binding sequence and that, although the putative IFN-αβ response element may have been involved in down-regulation of CAT expression, it was not the sole element responsible.

These comparative data are represented graphically in **figures 7.4 - 7.5.**

Figure 7.4. Comparison of IFN-γ and IFN-αβ effects on CAT expression between pDRα680CAT and pDRα320CAT-transfected U138MG

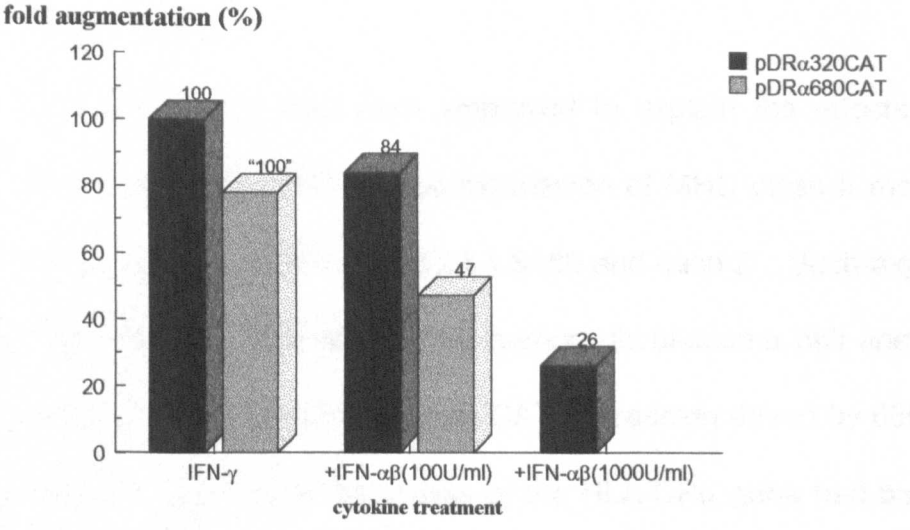
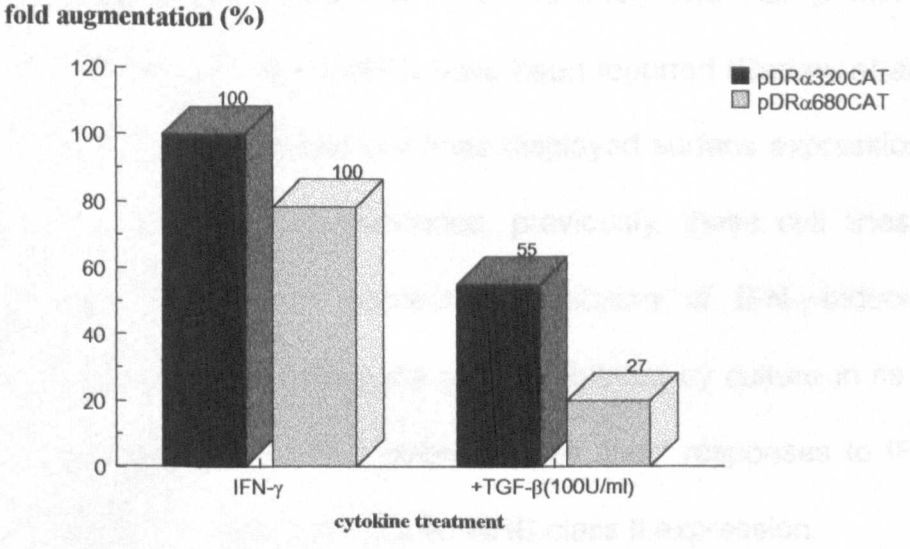


Figure 7.5. Comparison of IFN-γ and TGF-β effects on CAT expression between pDRα680CAT and pDRα320CAT-transfected U138MG



Notes:

The relative augmentation of CAT from the vectors pDRα320CAT and pDRα680CAT was compared after stimulation with IFN-γ alone or after costimulation of transfected U138MG with IFN-γ and IFN-αβ or with IFN-γ and TGF-β. Results are expressed as a percentage of the fold augmentation observed with IFN-γ alone (100%). The lengths of "IFN-γ" bars represent the differences in IFN-γ-induced CAT expression observed between these two vectors.

7.3. Transfection of human colorectal cell lines with reporter gene vectors

7.3.1. Introduction

Reporter gene assays were employed to explain the effects of IFN-γ, IFN-αβ and TGF-β upon the surface expression of MHC class II molecules in the colorectal cell lines colo 205, HT29, LS180 and caco 2 . Such experimental strategy had proved successful in the human glioblastoma cell line U138MG and the effects of these cytokines upon CAT expression driven by 680 and 320 base pair proximal promoter fragments of the HLA-DRα gene had been shown to be affected by deletion of a 360 base pair upstream sequence.

The responses of these cell lines to IFN-γ and TGF-β with regard to surface expression of class II MHC have been reported (Darley, *et al.*, (1993)). None of the colorectal tumour cell lines displayed surface expression of these antigens constitutively. As mentioned, previously, these cell lines were not responsive to TGF-β with respect to inhibition of IFN-γ-induced HLA-D expression although colo 205 were growth-inhibited by culture in its presence. No data were available for the colorectal cell lines' responses to IFN-αβ with respect to down-regulation of induced MHC class II expression.

The transfection of these cell lines with pDRα680CAT had not been successful in providing information regarding the cytokine-mediated control of MHC class II expression at the genetic level. It was determined that the 680 base pair HLA-DRα promoter fragment employed previously in CAT assays

either contained a tissue-specific repressor sequence or a lacked a tissue specific enhancer element.

The use of the pDRα320CAT vector in a series of experiments employing the same strategy as described previously was intended to determine which of the above possibilities for the unresponsiveness of the colorectal cell lines for HLA-DRα promoter-driven CAT expression was the most likely to be true.

7.3.2. Expression of reporter genes in colorectal tumour cell lines

7.3.2.1. Transfection of cells and treatment with cytokines

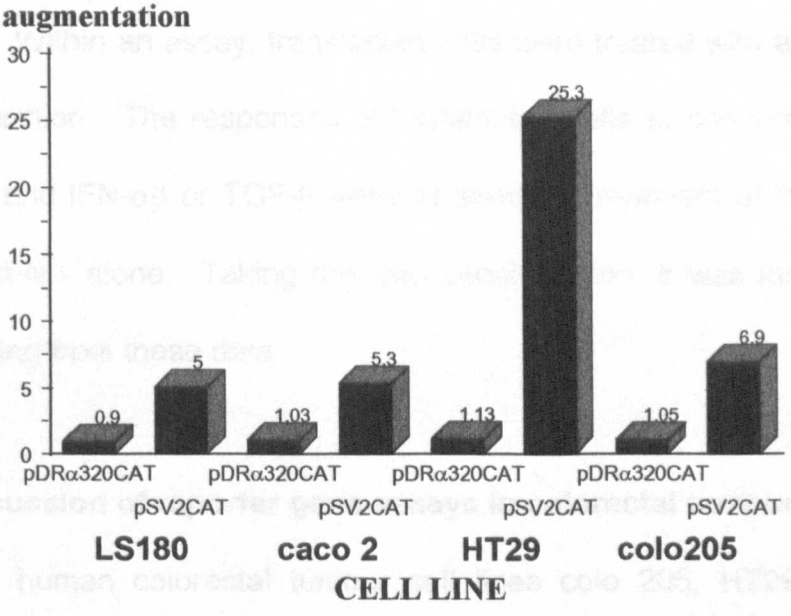
Colorectal tumour cell lines were transfected with reporter gene-containing vectors (pDRα320CAT, pSV2CAT and pRSVβ-gal) in the same proportions and by the same method as that described for the transfection of U138MG cells.

7.3.2.2. CAT expression by IFN-γ-treated, pDRα320CAT-transfected colorectal tumour cell lines

The response of pDRα320CAT-transfected cells to a range of IFN-γ concentrations was negligible as was the case when the same cell lines were transfected with pDRα680CAT and treated with the same cytokine concentrations. Transfected cells were treated with a range of IFN-γ concentrations from 1 to 100U/ml and no increase in CAT expression was observed in any of the cell lines over this range. Again, this inability of these cell lines to express the HLA-DRα promoter-driven expression of CAT was

irrespective of their transfection with and effective expression of both pSV2CAT and pRSVβ-gal. The levels of CAT expression from pDRα320CAT- and pSV2CAT-transfected cells are shown in **figure 7.6.**

Figure 7.6. Relative expression of CAT by colorectal tumour cell lines transfected with pDRα320CAT and pSV2CAT



Notes:

The colorectal tumour cell lines LS180, caco 2, HT29 and colo 205 were transfected with the vectors pDRα320CAT and pSV2CAT. CAT expression was induced by 100U/ml IFN-γ for 48hr and "pDRα320CAT" axis labels refer to the fold expression compared to untreated transfected cells. Constitutive CAT expression due to pSV2CAT (fold difference from pCATbasic) is identified by the axis label "pSV2CAT".

7.3.2.3 Effects of IFN-αβ and TGF-β treatment on CAT expression

Attempts were made to assess the effects of these cytokines upon the IFN-γ-induced CAT expression from the pDRα320CAT vector in the colorectal tumour cell lines. The information obtained from such data would have been

useful in the interpretation of the observations from the cell surface expression of HLA-D antigens by these cell lines under the influence of the cytokines studied here.

As was the case when these cells were transfected with pDRα680CAT, their response to IFN-γ with respect to expression of the reporter gene was negligible. Within an assay, transfected cells were treated with all cytokines in a "blind" fashion. The responses of transfected cells to concurrent treatment with IFN-γ and IFN-αβ or TGF-β were as seen for treatment of the transfected cells with IFN-γ alone. Taking this into consideration, it was inappropriate to infer anything from these data.

7.3.3. Discussion of reporter gene assays in colorectal tumour cell lines

The human colorectal tumour cell lines colo 205, HT29, LS180 and caco 2 were employed in reporter gene assays with the vector pDRα320CAT. Expression of the bacterial gene for chloramphenicol acetyl transferase was under the control of a 320 base pair proximal promoter fragment from the HLA-DRα gene. The expression of CAT in the appropriately transfected cells was attempted to be induced by their treatment with a range of IFN-γ concentrations. These cell lines did not express chloramphenicol acetyl transferase after treatment with any concentration of IFN-γ upto 100U/ml. This lack of inducibility was also observed when these colorectal tumour cell lines were transiently transfected with the vector pDRα680CAT. Again, this lack of

HLA-DRα promoter-driven CAT expression was irrespective of evidence of their transfection, from the expression of pRSVβ-gal. These cell lines also had the ability to process CAT transcripts into the functional enzyme as observed by their ability to express chloramphenicol acetyl transferase from pSV2CAT.

7.4. Discussion

7.4.1. Reporter gene assays with pDRα320CAT in U138MG cells

The human multiform glioblastoma cell line U138MG was employed in further investigations of HLA-DRα promoter-driven gene expression involving the reporter gene expression vector pDRα320CAT. The effects of the cytokines IFN-γ, IFN-αβ and TGF-β upon CAT expression were studied in cells transiently transfected with this vector. Levels of expression were compared to those observed for pDRα680CAT-transfected U138MG under the same cytokine stimuli.

Levels of IFN-γ-induced CAT expression were marginally greater for pDRα320CAT-transfected cells. This suggested that this deletion of the HLA-DRα promoter had removed an inhibitory sequence element located in the upstream 360 base pair region of the HLA-DRα680 fragment.

The suppression of IFN-γ-induced CAT expression in pDRα680CAT-transfected cells which had been treated concurrently with IFN-αβ, was virtually abolished when pDRα320CAT-transfected cells were treated with 1U/ml IFN-γ and 100U/ml IFN-αβ. Levels of inhibition equivalent to those described for pDRα680CAT-transfected cells under the stated cytokine concentrations were achieved here when the transfected cells were treated with 1U/ml IFN-γ concurrently with 1000U/ml IFN-αβ. The removal of the putative IFN-αβ response element had an effect on the ability of this cytokine to inhibit

the IFN-γ-induced expression of CAT. However, this was not the sole element responsible due to the restoration of inhibition when treated with the greater IFN-αβ concentration.

The inhibition of CAT expression by TGF-β was observed in pDRα320CAT-transfected cells and was evident when cells were treated concurrently with 1U/ml IFN-γ and 100U/ml TGF-β. However, this down-regulation of expression was not as great as when pDRα680CAT-transfected cells were treated with the same concentrations of these cytokines.

In conclusion, results obtained with pDRα320CAT-transfected U138MG cells indicated that a general repressor element had been removed from the promoter by the deletion of the 360 base pairs of the upstream portion of the HLA-DRα680 promoter fragment.

This region contained a sequence with homology to an IFN-αβ response element and it was considered that this sequence was responsible for mediation of the repression of IFN-γ-induced expression by IFN-αβ. However, due to the greater levels of IFN-γ-induced CAT expression and the reduced inhibition of this expression by TGF-β as well as by IFN-αβ, it was postulated that a more general repressor element was responsible for overall down-regulation of gene expression from this promoter instead of or as well as the proposed IFN-αβ sequence element.

The promoter sequence responsible for TGF-β-mediated suppression was contained within the 320 base pair HLA-DRα promoter fragment.

These results were in agreement with those of Reimold *et al.*, (1993) in the melanoma cell line Hs294T(c) and Devajyothi *et al.*, (1993) in the CRT astrocytoma cell line. The latter study suggested that TGF-β suppression of HLA-DRα expression was not due to global transcriptional inhibition in that the IFN-γ-induced expression of the intercellular adhesion molecule-1 (ICAM-1) was unaffected by TGF-β. Mutational analysis of the HLA-DRα promoter by Reimold *et al.*, resulted in the conclusion that TGF-β exerts its effects through sequences within the W-X_1 region of the promoter. Repression due to IFN-β was also shown to be exerted through the 267 base pairs proximal to the transcriptional start site and this repression of CAT expression was shown to be less than displayed by a 2.2Kb HLA-DRα heterologous promoter (Devajyothi *et al.*, 1993). These results reflected those obtained in the present study and indicated also that repression by TGF-β was reduced in the truncated promoter constructs in addition to the increased levels of IFN-γ-induced CAT expression by cells transfected with such plasmids.

Recent studies have speculated as to the cellular and molecular mechanisms for the down-regulation of IFN-γ-induced class II MHC expression. Dose and time dependent inhibition of expression of class II surface protein, mRNA, and promoter activity by TGF-β has been demonstrated in primary astrocytes (Panek, *et al.*, 1995). In this study, evidence was provided for the specific inhibition of transcription of MHC class II rather than a global effect. Furthermore, TGF-β did not affect events that are involved in IFN-γ-induced intracellular signalling such as tyrosine phosphorylation of JAK1, JAK2 and STAT1α, nor did it affect IFN-γ induction of class II X_2 box binding protein IFNEX.

It was speculated that TGF-β may exert its effects by modulation of the expression or function of constitutively expressed factors responsible for regulation of class II MHC gene expression.

The current studies into the down-regulation of class II MHC expression are clearly at a preliminary stage, but evidence from this investigation and those presently being undertaken are revealing important aspects of the control of transcription of these genes. As yet, there are few published data with respect to the molecular mechanisms of IFN-αβ-mediated inhibition of IFN-γ-induced class II MHC expression, but those available support the observations recorded here (Devajyothi *et al.*, 1993). There has been no published observation of an IFN-αβ or a

TGF-β-induced repressor factor binding to the HLA-DRα promoter either in the presence or absence of concurrent treatment of cells with IFN-γ. However, there was observation made in the present investigation that IFN-αβ treatment of the colorectal tumour cell lines colo 205 and LS180 resulted in increased protection of the W-X box region of the HLA-DRα promoter. Again, this was an unsubstantiated observation which clearly requires further investigation. Also, no such experiment was possible with the U138MG cell lines due to technical difficulties in obtaining nuclear proteins by the method described.

7.4.2. Reporter gene assays with pDRα320CAT in colorectal tumour cell lines

An attempt was made to relate observations of the surface expression of HLA-D antigens in response to cytokine treatment to the structure of the HLA-DRα promoter. The inability of the human colorectal tumour cell lines caco 2 and LS180 to express class II MHC antigens in response to stimulation by IFN-γ was of particular interest. *In vivo* footprinting and complementation studies involving HLA-DRα expression in non-inducible cell types have determined that promoter accessibility within the MHC is an important factor in the transcription of these genes (Kara, 1993). This type of reporter gene study was intended to clarify the status of the non-inducible colorectal cell lines in this regard. Whether their uninducibility for the expression of class II MHC antigens was a result of promoter structure not permitting organisation of the transcriptional machinery would have been revealed should they have

expressed chloramphenicol acetyl transferase driven by the HLA-DRα promoter.

As was the situation with the vector pDRα680CAT, none of the colorectal tumour cell lines studied here were capable of expressing CAT when transfected with pDRα320CAT. This lack of expression was again irrespective of the cell lines' abilities to express CAT when driven by the SV40 late promoter and was not explained by the cells not being transfected efficiently with the vector. These observations remain fully unexplained although the ability of these cells to respond to IFN-γ with respect to induction of other genes post transfection was not assessed.

These collective observations led to the postulate that uninducibility of CAT from either of the HLA-DRα promoter vectors employed was most likely to be the result of a missing tissue-specific enhancer element. This hypothesis could have been tested by a series of reporter gene assays employing larger HLA-DRα promoter fragments.

CHAPTER 8

CHAPTER 8: DISCUSSION

The experimentation described in this report was performed with the intent of understanding further the mechanisms involved in the cytokine-mediated induction and inhibition of Class II MHC gene expression in human tumour cell lines. The following sections relate the results obtained in this investigation with published data and offer an opinion of their possible implications for the control of the expression of HLA-DRα.

8.1. DNA binding activities within nuclear extracts from inducible and non-inducible tumour cell lines

Sequence elements important for the transcriptional regulation of HLA-DRα gene expression were investigated in bandshift (gel retardation, electromobility shift) assays as to their abilities to bind nuclear factors from human tumour cell lines prior to and after IFN-γ stimulation. The *cis*-acting elements chosen for this were the W and X boxes which had been shown to be important for IFN-γ-stimulated expression of class II MHC genes (Basta, *et al.*, 1988; Tsang, *et al.*, 1990; Sherman, *et al.*, 1987, 1989). The primary objective of this experimentation was to determine whether there were differences in the populations of HLA-DRα promoter element binding proteins between inducible and non-inducible human tumour cell lines. Also important here was the effect of IFN-γ treatment of cells on the binding of such transcription factors. Though

much research over some years has concentrated upon the IFN-γ-stimulation of HLA-DRα gene expression, no IFN-γ-induced HLA-DRα promoter-associated transcription factor has been identified. A probe corresponding to the whole promoter region was also employed in this type of assay.

8.1.1. The HLA-DRα W box

The binding of transcription factors from uninduced and IFN-γ-induced human tumour cell lines to a probe corresponding to the HLA-DRα W box revealed cell line- and cytokine treatment-dependent differences in the proteins which bound prior to IFN-γ-treatment of the cells.

The cell lines studied contained different populations of W box binding proteins. It was not possible to equate the presence of the complexes observed with those published by other authors who have described the presence of complexes constitutively present in nuclear extracts from Raji, Jurkat and HeLa cells. In lymphoid cells such as Raji, the W box-binding complexes W-B1 and W-B2 have been described (Sakurai & Strominger, 1988; and Tsang *et al*, 1990). While W-B1 is ubiquitous , W-B2 is a lymphoid-cell-specific protein (Cogswell, *et al.,* 1991a). Of these two proteins, W-B2 displays the greatest mobility in bandshift assays. It was difficult to assign these labels to any of the bands obtained with the cell lines studied in this investigation.

The availability of antisera to W-B1/2 at the time of investigation would have enabled "supershift" or competition assays to have been performed. The

binding of an antibody to a DNA-associated factor results in further inhibition of the complex's electrophoretic migration compared to the same protein-DNA complex in the absence of antibody. Alternatively, an antibody with specificity for the DNA binding site of a transcription factor could be utilised to block the association of a factor with its recognition sequence.

Upon IFN-γ-treatment of the class II-inducible cell lines colo 201, colo 205 and U373MG, an altered population of W box-binding transcription factors was observed. In colo 201 and colo 205, this was manifested in the diminution of complexes as well as the binding of a novel factor which migrated with the same mobility for both cell lines. The novel complex migrated with similar, but not the same, properties as the most retarded band observed in untreated and IFN-γ-treated LS180 and caco 2. It was postulated that the novel binding complex in colo 201 and colo 205 was formed by the alteration of a previously bound factor(s).

The modification of transcription factors upon stimulation of intracellular signalling mechanisms is noted. For example cyclic AMP mediates the hormonal induction of numerous eukaryotic genes through a conserved cAMP response element (CRE). A rat somatostatin promoter CRE-binding phosphoprotein was identified which is modulated by cAMP-dependent protein kinase-mediated phosphorylation (Yamamoto, *et al.,* 1988). In this situation phosphorylation of CREB allows its dimerisation and the dimer is observed as a more retarded complex than the monomer in gel retardation assays.

For IFN-γ-induced expression of HLA-DRα, it has been shown that cytokine stimulation is associated with activation of protein kinase C in astrocytes (Benveniste, *et al.,* 1991; Lee, *et al.,* 1995). It might, therefore, be postulated that covalent modification of transcription factors is important for IFN-γ-mediated stimulation of class II MHC expression. If the most retarded complex observed with uninduced colo 201 and colo 205 became modified upon IFN-γ stimulation then its electrophoretic properties may be altered also.

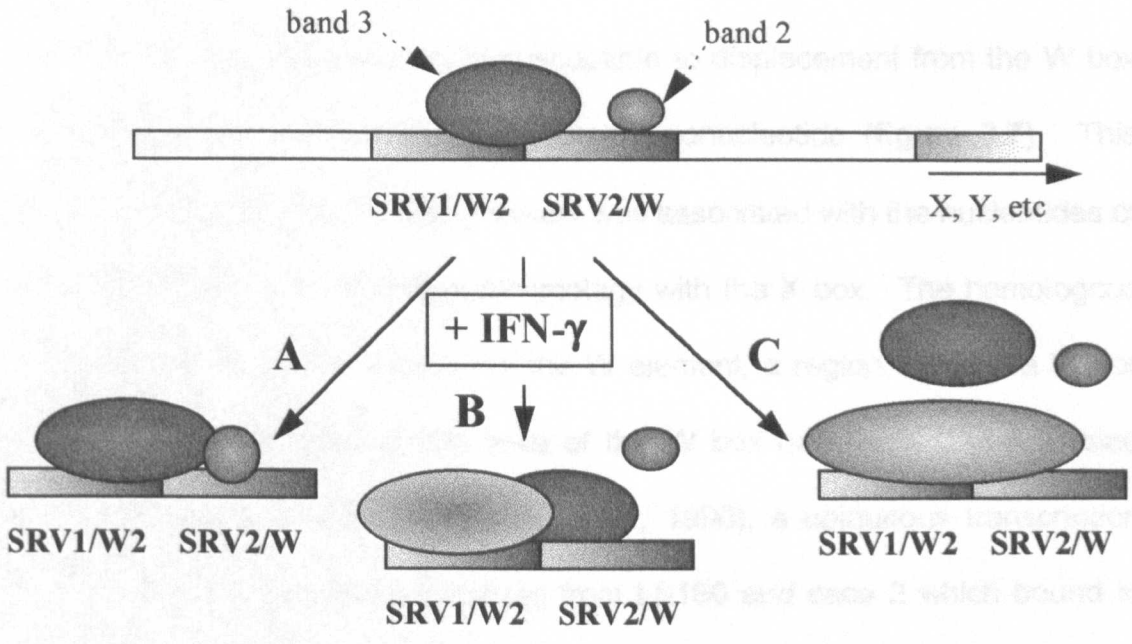
The appearance of the "novel" retarded complex upon IFN-γ treatment of colo 201 and colo 205 was correlated with the reduced association of two factors bound prior to stimulation. In colo 205 the binding of the fastest migrating of these uninduced complexes was nearly completely abolished upon treatment of cells for 6 hr with IFN-γ, and similarly after 24 hr treatment. The novel complex observed with this cell line was only observed after 24hr IFN-γ treatment. This suggested that the dissociated complex may have been exerting a repressive effect at this site which was relieved upon cytokine stimulation and that only after dissociation of this factor could the novel complex bind. However, in colo 201 the association of the IFN-γ-induced complex was irrespective of the presence of this faster migrating "diminished" complex.

Three postulates could be drawn from these observations of the IFN-γ-stimulation of these colorectal tumour cell lines. Firstly, it was hypothesised that the novel retarded complex induced by IFN-γ treatment was formed from two ubiquitously-bound factors which became modified upon

cellular stimulation. The contributory factors were identified as those whose intensity was reduced along with the binding of the induced complex after 6 and 24hr IFN-γ treatment in colo 201. Secondly, a hypothesis could be constructed in which the binding of a modified factor is permitted after the dissociation of a repressor complex. This postulate might be applied to the IFN-γ treatment of colo 205 where the binding of the induced complex was only observed after considerable reduction in the intensity of the complex which gave rise to "band 2" (**figure 3.3**). Similarly, the association of the IFN-γ-induced complex observed with colo 201 may also be explained by these considerations. Thirdly, the novel complex may not be a factor which resulted from the covalent modification of a protein bound prior to IFN-γ treatment, but a new species of protein. In this scenario the diminution of factors upon IFN-γ treatment is due to their displacement by the induced factor. These hypotheses are represented schematically in **figure 8.1.**

In the uninducible cell lines LS180 and caco 2 there was no alteration in the binding of factors after IFN-γ treatment. In addition, the most retarded complex from these cell lines migrated with similar, but slower mobility than the induced factor from IFN-γ-stimulated colo 201 and colo 205. The binding of the factor giving rise to "band 3" in colo 201 and colo 205 was disabled in the uninducible cell lines. This factor may therefore have an important rôle in the IFN-γ-induced transcription of HLA-DRα through the W box sequence element.

Figure 8.1. Hypotheses of the IFN-γ-induced binding of transcription factors to the HLA-DRα W box probe in colo 201 and colo 205



Three possible scenarios for the IFN-γ-induced binding of transcription factors to the HLA-DRα W box probe are indicated. Prior to IFN-γ treatment of colo 201/205, two major binding species are depicted corresponding to bands 2 and 3 in figure 3.3. The W box is dissected into the W (SRV2) and W2 (SRV1) elements. **A)** IFN-γ treatment results in covalent modification of bound factors and their altered association with the element. **B)** A "repressor" factor (band 2) dissociates from the W box and a modification of "band 3" results in its altered association with the element. **C)** The binding of an IFN-γ-induced binding factor results in the dissociation of the previously bound proteins.

Competition for the binding of W-box bound factors by an X box oligonucleotide indicated that homologous sequences within these elements were targetted by W box-specific factors. The IFN-γ-induced factor from colo 201 and colo 205 was particularly susceptible to displacement from the W box by the presence of the X box competitor oligonucleotide (**figure 3.7**). This indicated that the induced binding moiety was associated with the nucleotides of the W box which showed greatest homology with the X box. The homologous nucleotides are at the 5' extreme of the W element, a region called the W2 or SRV1 element. Previously, this area of the W box had been shown to bind W-B1 (Tsang, *et al.,* 1990; Cogswell, *et al.,* 1990), a ubiquitous transcription factor. The most retarded complexes from LS180 and caco 2 which bound to the W box were also partially competed out by the presence of the X box oligonucleotide. This crude method, therefore, localised the colo 201/205 induced factor and the LS180/caco 2 constitutive factor to the 5' region of the W box.

In the inducible astrocytoma cell line U373MG there was also novel binding observed upon IFN-γ treatment of cells. Again this was manifested in the association of a slowly-migrating complex and a complex which migrated with comparatively greater mobility (figure 3.2). In this instance, there was no concurrent loss of binding of previously observed factors. On the contrary, there was an increase in the association of all factors in addition to those which bound upon IFN-γ stimulation.

8.1.2. The HLA-DRα X box

This sequence element was assessed for the binding of nuclear factors in the same manner as that described for the W box. Again, differences were observed between the inducible colorectal tumour cell lines and those which were uninducible for class II MHC expression. This was manifested in the presence of protein-DNA complexes which were similar for the uninducible lines, yet those observed for the unstimulated, inducible cell lines were markedly different. As for the association of proteins with the W box after IFN-γ treatment there were differences in the binding of factors to the X box upon stimulation of the inducible colorectal cell lines colo 201 and colo 205. Common to both of these cell lines was the appearance of a low-mobility complex with the same migratory properties as the most retarded complex from LS180, similarly with caco 2. In colo 201, a second induced factor was also observed, this complex had greater mobility than the other induced factor and no factor with its migratory properties was displayed by the other cell lines, ie, this factor was unique to IFN-γ-induced colo 201. In colo 205, the binding of the induced factor was coincident with the dissociation of two complexes observed prior to IFN-γ-stimulation.

The involvement of the described X box-binding factors RF-X and hXBP-1 in the formation of the observed complexes could not be assessed at the time of investigation. RF-X is a large protein and has been reported to give rise to two X box-binding moieties in bandshift assays, termed the B1 (slower mobility) and B3 (faster mobility) (Cogswell *et al,* 1991b). The RF-X which

gave rise to the faster migrating B3 band was reported to have a DNase I footprint covering the 5' region of the X box and a few upstream nucleotides. However, the slower migrating B1 RF-X complex also made additional contacts in the 3' Y element and with bases -126 and -127 within the upstream W element; competition analyses have indicated that nucleotides within the W box a required for stabilising the binding of the B1 band RF-X (Reith, *et al.,* 1988). With regards to the bandshift employing colorectal cell line nuclear extracts, it was possible that band 4 was attributed to the binding of RF-X to the X box nucleotides and that band 5, most noticeably observed in extracts from caco 2, was due to the association of W and Y box-binding proteins with RF-X resulting in a retarded band. It is of note that, assuming band 4 was caused by the binding of RF-X, this protein was absent from the nuclear extracts of unstimulated colo 205 and was only present in small amounts in those of untreated colo 201 and caco 2. The use of this terminology is in a purely postulative manner in the absence of evidence from supershift-type experiments, yet it is of use to explain speculatively the binding of the observed factors to the X box.

Interferon-γ, promoted a response in the MHC class II inducible cell lines colo 201 and colo 205, but not U373MG, as regards the proteins which bound to the X box of the HLA-DRα promoter. In unstimulated colo 201, a band which may be postulated as being due to the presence of RF-X was only bound to the X box in small amounts and this binding was very much increased by the treatment of the cells with IFN-γ for 6hr. Such stimulation also resulted in the

binding of a completely novel factor to this region (termed band A).

The altered binding of factors from IFN-γ-stimulated colo 201 to this probe may be explained by a number of hypotheses. Firstly, this factor may not be present or is only present at low levels in the unstimulated cells; treatment with IFN-γ leads to increased copy number by transcriptional regulatory mechanisms. A second hypothesis might suggest that the factor is constitutively present in the nuclei of untreated cells, but it is in a form which cannot bind efficiently to the X box; modification of the protein upon IFN-γ treatment of the cells results in a different form which is able to bind to the X box. Thirdly, it is possible that the binding of the factor is assisted by the binding of a protein cofactor. This is supported by the additional binding of a second complex with the colo 201 extracts (band A).

In the case of colo 205, a fourth possibility was presented. Here, it was possible that the binding of the novel complex is inhibited by repressor proteins which are bound to the X box in unstimulated cells (bands 2 and 3). Treatment of the cells with IFN-γ results in their displacement by the induced complex and transcription is activated. In nuclear extracts from LS180 and caco 2 (with and without IFN-γ treatment) a factor with the same mobility as the most retarded induced factor in colo 201/205 is constantly present as is the complex which gives rise to band 2, the postulated repressor. It is possible therefore, that transcription is inhibited by the constitutive presence of this factor in these cell lines which cannot be induced to express class II MHC antigens. A schematic

representation of the hypotheses for the IFN-γ-induced binding of the induced factor(s) is given in **figure 8.2.**

Figure 8.2 Models for the interferon-γ-activation of transcription through the X box

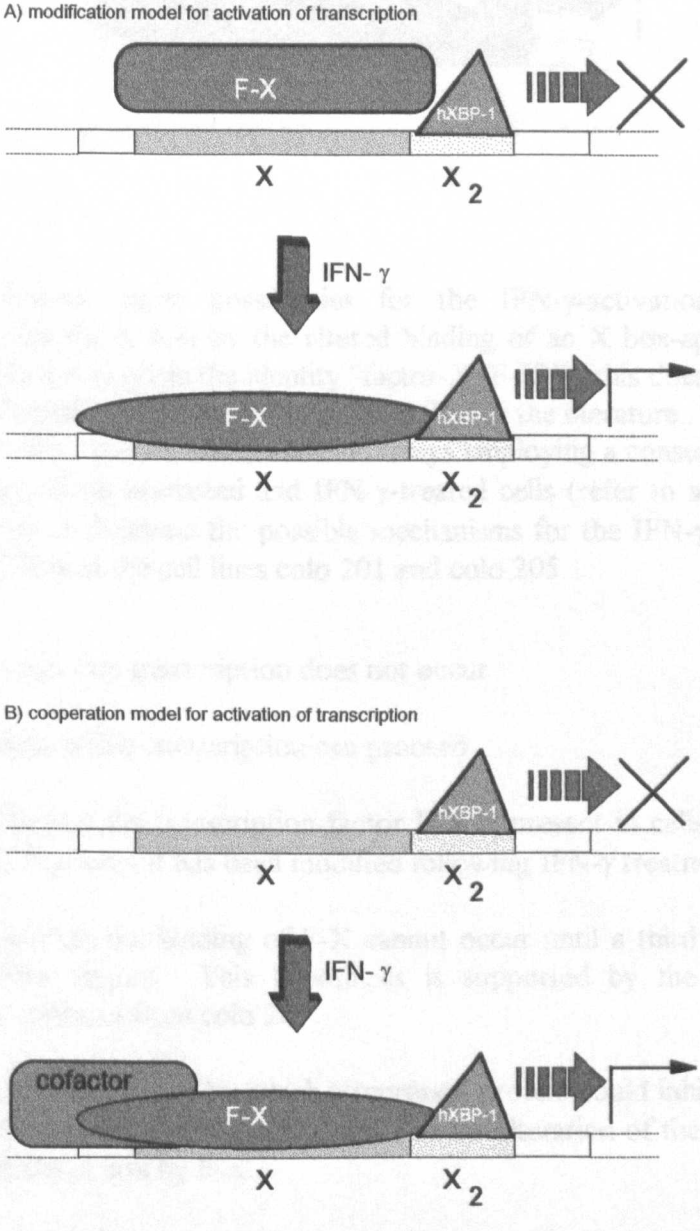
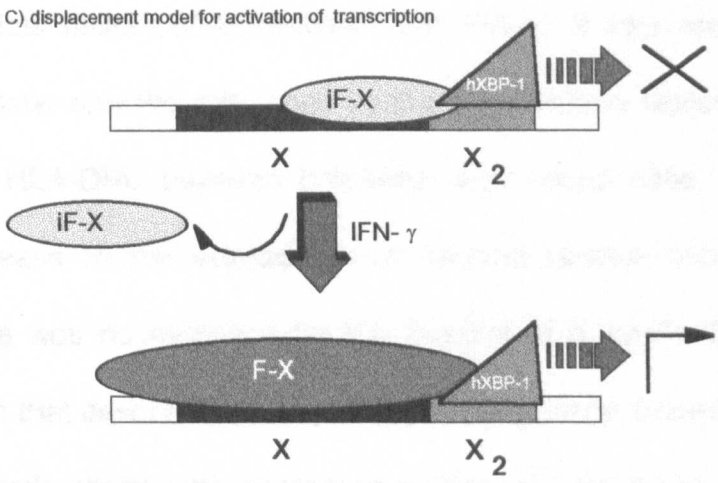
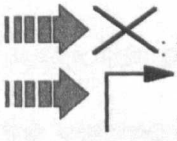


Figure 8.2. (contd.)



Notes:
The diagrams illustrate three possibilities for the IFN-γ-activation of HLA-DRα transcription through the X box by the altered binding of an X box-specific factor. In these figures, this factor is given the identity "factor-X (F-X)" - this does not indicate that this has been confirmed as the same RF-X as described in the literature.. The mechanisms shown are devised from results from bandshift assays employing a consensus X box probe and nuclear extracts from untreated and IFN-γ-treated cells (refer to section 3.3). The models are intended to illustrate the possible mechanisms for the IFN-γ-induced binding of factors to the X box in the cell lines colo 201 and colo 205.


: indicates that transcription does not occur

: indicates that transcription can proceed

Model A) suggests that the transcription factor F-X is present in cells but is unable to interact with the X box until it has been modified following IFN-γ treatment of cells.

Model B) indicates that the binding of F-X cannot occur until a third party factor also binds to the X box region. This hypothesis is supported by the results obtained employing nuclear extracts from colo 205.

Model C) shows the mechanism by which a repressor protein could inhibit the binding of F-X and that IFN-γ treatment of the cells results in the alteration of the repressor and its displacement from the X box by F-X.

The binding of the factor human X box binding protein 1 (hXBP-1) is shown as constitutive.

The astrocytoma cell line U373MG did not display any difference in the binding of nuclear factors after treatment with IFN-γ. It was assumed that this displayed a tissue-specific difference in the transcription factors required for IFN-γ-induced HLA-DRα between colorectal and neural cells. There was a moderate increase in the association of existing factors with this element. However, there was no evidence for the binding of a single IFN-γ-enhanced factor similar to that described in rat primary astrocytes by Moses, *et al.*, (1992). These neural cells presumably contained all the required X box-binding factors irrespective of IFN-γ treatment and such treatment was responsible for changes at the W box instead.

8.1.3. The HLA-DRα470 promoter probe

This probe represented the region of the HLA-DRα promoter form nucleotide positions -423 to +47 relative to the cap site. Nuclear extracts from the colorectal tumour cell lines colo 205, LS180 and caco 2 were employed in the investigation of the binding of transcription factors to the fragment probe. The effect of IFN-γ treatment of cells was assessed and preliminary experiments investigated the effects the concurrent treatment of cells with IFN-γ and IFN-αβ.

Constitutive binding of nuclear factors

Differences were observed in the binding of transcription factors to this promoter probe between the colorectal tumour cell lines, although these

differences were not as marked as those observed with oligonucleotide probes. One prominent band was observed for all cell lines, other bands varied with cell line and cytokine treatment. Again, the major difference was between the inducible colo 205 and the other two non-inducible lines. This was manifested in the presence of a slowly migrating complex which formed the major binding activity in colo 205 which was not present with LS180 or caco 2.

The use of competition bandshift assays, employing W, X and Y box oligonucleotides as competitors, resolved the slowest migrating complex of colo 205 into two factors which bound independently to the W and X boxes and that the majority of binding of factors was at the X box region. It was also shown that the "invariant" band's intensity was altered, although it was never completely abolished, by all combinations of competitor oligonucleotides. It was possible that this band was due to the binding of factors to the W, X and Y elements, that is to say that the association of many factors with the promoter resulted in one major nucleoprotein-DNA complex observed by this method. The incorporation of competitor oligonucleotides resulted in differences in intensity of this intermediately migrating complex and the appearance of a rapidly migrating complex in colo 205 and LS180 whose intensity remained unaltered by competition. The sequestration of constitutively bound W, X and Y box-associated factors, therefore, allowed the binding of a high mobility complex whose binding was not affected by the presence of competitor oligonucleotides. It was postulated that this factor may have been a repressor protein or a factor which bound to the promoter when the numbers of bound

transcription factors was low. The rôle of such a factor might be to maintain promoter structure in a "poised" manner until the stimulus for the binding of more transcription factors. The function of the "repressor" factor might well be a structural one such that it maintains the structure of the promoter in conditions where the constitutive promoter binding proteins are not present or are not present insufficient concentration. Binding of such a factor would allow the promoter to be poised for the association of transcription factors with their corresponding sequence elements once the concentration of the transcription factors is great enough to displace the "repressor" factor. *In vivo* footprinting data have suggested that class II MHC inducible non-lymphoid cells possess a "poised" promoter in the absence of IFN-γ (Wright & Ting, 1992) It was proposed that the majority of protein-DNA interactions are in place prior to IFN-γ stimulation but with certain interactions being weak and non-productive. Interferon-γ stimulation was proposed to modify existing DNA binding factors leading to a stronger and productive interaction with the promoter, or such stimulation could cause *de novo* binding of an induced factor.

Binding of nuclear factors after IFN-γ treatment of cells

The treatment of colo 205 with IFN-γ resulted in the disappearance of the most retarded complex(es) which had been shown to bind to the W and X boxes prior to stimulation. Again, therefore, a difference was observed in the binding of complexes to a probe after IFN-γ treatment of an inducible cell line. In this situation, it was manifested in the reduction in binding of proteins. This had also been the case when the W and X box oligonucleotides were used as

probes with extracts from this cell line, although there had also been induced binding of factors by upon IFN-γ treatment in these instances.

The treatment of colo 205 with IFN-γ also resulted in the absence of binding of the fastest migrating repressor/maintenance factor which had been observed when untreated nuclear extracts were incubated with probe and competitor oligonucleotides.

Treatment of the uninducible cell lines LS180 and caco 2 with IFN-γ resulted in no differences in the binding of factors to this probe. Also, the fastest migrating complex was still observed in IFN-γ-treated LS180. If this complex did represent a "repressed-state" promoter maintenance factor then it was present in LS180 nuclear extracts irrespective of stimulation - perhaps indicating the inability of this cell line to transduce the IFN-γ signal into the binding of transcription factors to this promoter. Conversely, this complex was not observed with caco 2 under any circumstances. If the postulate is to be adhered to then it could be said that this cell line lacked a nuclear factor which was responsible for the organisation of constitutively bound transcription factors in readiness for stimulation of gene expression.

8.1.4. DNase I footprinting of the HLA-DRα 470 probe

This technique was employed to clarify the data obtained from bandshift experiments such that contact points of transcription factors could be identified

and any differences in the contact points of apparently similar factors observed in bandshifts could be determined. As discussed in chapter 5, there were many technical difficulties encountered with this type of experiment and the production of a clear footprint of the HLA-DRα470 probe was hard to come by. When this probe was successfully footprinted, protected regions of the probe were observed which corresponded to defined sequence elements of the HLA-DRα promoter. There was no observed difference in the protected regions between the cell lines, nor was there any difference with IFN-γ-treated colo 205 nuclear extracts compared to unstimulated cells. However, when nuclear extracts from IFN-αβ & IFN-γ costimulated colo 205 and LS180 resulted in an increased protection at the W-X box region, the appearance of a novel binding factor in the corresponding bandshift was not observed. This increased protection was seen as the result of the proteins already bound to the W and X boxes altering their contacts with the promoter under the IFN-αβ/IFN-γ costimulus.

8.2. Functional analyses of the HLA-DRα promoter: inhibitory effects of IFN-αβ and TGF-β on IFN-γ-induced gene expression

8.2.1. Reporter gene assays with HLA-DRα 680 promoter fragment

CAT reporter gene assays employing the glioblastoma cell line U138MG transiently transfected with the vector pDRα680CAT demonstrated the effects of IFN-γ, IFN-γ/IFN-αβ and IFN-γ/TGF-β upon gene expression driven by a 680bp fragment of the HLA-DRα promoter. This cell line was used after what had seemed to be a failure of transfection and/or subsequent induced reporter gene expression in similar experiments in the colorectal tumour cell lines employed in the studies of DNA binding proteins.

In U138MG, it was shown that 1U/ml IFN-γ could stimulate near maximal expression of chloramphenicol acetyl transferase driven by the heterologous HLA-DRα promoter fragment. This fragment contained all those elements shown to be essential for IFN-γ-induced expression (see **section 1.6.3.**) as well as a consensus IFN-αβ response element. The treatment of U138MG transiently transfected with pDRα680CAT with IFN-αβ or TGF-β as well as IFN-γ resulted in the induced expression of CAT being much reduced compared to those transfected cells treated with IFN-γ alone (chapter 6).

When the assay technique was reassessed in the colorectal tumour cell lines, no CAT expression from the pDRα680CAT plasmid was observed even

though CAT expression was clearly evident in cells transfected with a positive control vector, pSV2CAT.

It was concluded that, as demonstrated in U138MG, the HLA-DRα680 promoter fragment contained sequences responsible for the IFN-γ-induced expression of a reporter gene and also the IFN-αβ and TGF-β-mediated inhibition of this induction. The same promoter fragment was unable to direct induced expression of CAT in colorectal tumour cell lines. It was proposed that this promoter fragment either: a) possessed a tissue-specific repressor element which silenced this promoter fragment in these cell lines; b) lacked a tissue-specific enhancer element essential for gene expression directed by the HLA-DRα promoter in cells derived from the colon epithelium.

8.2.2. Reporter gene assays with the HLA-DRα 320 promoter fragment

The expression vector pDRα320CAT contained a 320bp promoter fragment which had been produced by the deletion of the 5' region of the 680bp fragment. The deleted portion of the promoter contained a consensus IFN-αβ response element which may have contributed to the inhibition of IFN-γ-induced expression of CAT driven by the 680bp promoter fragment.

When pDRα320CAT was employed in reporter gene assays with U138MG the relative expression of IFN-γ-induced CAT was greater than was observed with the pDRα680CAT vector.

The IFN-αβ inhibition of IFN-γ-induced CAT expression observed with U138MG when transfected with pDRα680CAT was not observed under similar circumstances when the cells were transfected with pDRα320CAT. The inhibition of expression by IFN-αβ was restored when 1000U/ml was used against 1U/ml of stimulating IFN-γ. It appeared, therefore, that there was an element which had been removed by truncation of the promoter which was involved in IFN-αβ-mediated inhibition of gene expression driven by the HLA-DRα promoter. However, this was not the critical element since inhibition was restorable with increased IFN-αβ concentration. It was proposed that the 320bp HLA-DRα promoter fragment was essential for IFN-αβ-mediated inhibition of CAT expression and that a sequence upstream of this enabled a more sensitive response.

The inhibition of IFN-γ-induced CAT expression mediated by TGF-β was also reduced in cells transfected with pDRα320CAT compared to those transfected with pDRα680CAT. However, this inhibition was still evident and it was again proposed that the 320bp promoter fragment was sufficient to direct TGF-β-mediated inhibition of expression.

The reduced inhibition of gene expression in cells transfected with pDRα320CAT, compared to that when expression was driven by the larger promoter fragment, was considered to be the result of the removal of a negatively acting *cis*-element. A candidate element was the consensus IFN-αβ

response sequence. However, it was also possible that a general repressor element had been removed due to the reduction in TGF-β effects and the increase in IFN-γ-induced expression.

Colorectal tumour cell lines were again assessed for their ability to express CAT driven by an HLA-DRα promoter fragment. It had been proposed that their inability to do so when expression was driven by the 680bp promoter fragment was because this fragment contained a cell-type-specific repressor element or that it lacked an enhancer element essential for gene expression. These postulates were assessed by the use of pDRα320CAT in reporter gene assays.

None of the colorectal tumour cell lines expressed CAT when driven by the deleted promoter fragment. Again, this was irrespective of their ability to express CAT when driven by the SV40 late promoter. It was therefore assumed that the fragment of the promoter removed in the construction of pDRα320CAT did not contain a tissue-specific repressor element. The possibilities remaining was that a tissue-specific silencer was present in the 320bp promoter fragment or that a tissue-specific enhancer was absent from this region. These hypotheses are yet to be tested experimentally.

8.3. Proposals for further research

This investigation, apart from fulfilling the main objectives, raised many questions which, due to the intensive nature of the research, have yet to be answered. The research described in this thesis was of a demanding nature, both technically and in terms of the time taken to complete certain experiments. However, the data described here were included only after repeated experiments had confirmed the result and, as such, are considered to be valuable.

As stated above, this research raised many questions which need to be addressed before a complete picture of the expression of HLA-DRα can be painted. The scope for further research is described below.

The first priority is to establish the identity of those factors which were shown to bind to the W and X box oligonucleotides. Antibodies to cloned HLA-DRα promoter-binding transcription factors were not available at the time this investigation was undertaken. Had they been, it would have been possible to determine which of those bound factors correlated with those which had already been identified and characterised. Those factors peculiar to the IFN-γ-inducible cell lines would also be attractive for further research. Their identification is central to answering those questions raised by the original research.

The involvement of IFN-γ-induced "cofactor" CIITA also needs attention. Some of the models for the binding of IFN-γ-induced factors to the W and X box probes indicated the possible involvement of a cofactor-type protein. The described cofactor has been shown to be necessary for IFN-γ-induced transactivation through the W (S) and X2 boxes (Zhou and Glimcher, 1995) where its association has been termed as the "on/off" switch for transcription. The actual interaction sites of CIITA with transcription factors bound to class II MHC promoters was demonstrated at the time of completion of this work. Again, the positive identification of CIITA as being one of the factors involved in IFN-γ-regulation of HLA-DRα transcription in the cell lines studied may rely on the availability of appropriate antiserum, or antisense RNA which might alter its binding in bandshift-type experiments or its presence in nuclear extracts from these cell lines.

Although *in vitro* DNase I footprinting of the HLA-DRα promoter revealed no differences in the binding of nuclear extracts between inducible and non-inducible cell lines, the analysis of bound factors *in vivo* was not assessed. This technique might be useful in future research since it displays the association of transcription factors *in situ* rather than the more artificial situation imposed by the *in vitro* technique.

The delineation of the region(s) of the HLA-DRα promoter responsible for the inhibition of IFN-γ-induced gene expression by IFN-αβ and TGF-β is yet to be concluded. Research has shown, through reporter gene assays, that

repression is mediated through the W-X-Y region of the promoter, although no sequence element has been described, nor has a DNA binding factor binding factor been identified. The identification of a sequence element responsible for inhibition would be difficult if the *cis*-acting sequences essential for IFN-γ-induced expression are also involved. The mutation of W, X or Y boxes abolishes expression of HLA-DRα so the inhibition of gene expression by IFN-αβ or TGF-β could not be assessed against this. This is the probable reason for no further publications regarding this matter since those which originally described the phenomenon.

Also interesting is the potential for a tissue-specific enhancer which is apparently required for HLA-DRα expression by colorectal tumour cell lines. The use of larger promoter fragments in reporter gene constructs or even "enhancer-trap" reporter gene constructs employing intronic sequences from the HLA-DRα gene would be useful in concluding the situation with this type of cell line.

Finally, this study has whetted the appetite for further research into a wider range of cell types. It would be interesting to establish whether differences described between inducible and non-inducible cell lines are translated to cells of different tissue origin.

BIBLIOGRAPHY

BIBLIOGRAPHY

Alam, J., and Cook, J. L., (1990). Reporter genes: application to the study of mammalian gene transcription. *Analytical Biochem.* **188:** 245-254

Albino, A. P., Houghton, A. N., Eisinger, M., Lee, J. S., Kantor, R. R. S., Oliff, A. I. and Old, L. J., (1986). Class II histocompatibility antigen expression in human melanocytes transformed by Harvey murine sarcoma virus (Ha-MSV) and Kirsten MSV retroviruses. *J. Exp. Med.,* **164:** 1710-1722.

Amaldi, I., Reith, W., Berte, C. and Mach., (1989). Induction of HLA class II genes by IFN-γ is transcriptional and requires a *trans*-acting protein. *J. Immunol.* **142:** 999-1004.

Bagli, D. J., D'Emilia, J. C., Summerhayes, I. C., Steele, G. D. and Barlozzari, T., (1990). C-Ha-*ras*-I oncogene-induced differentiation and natural killer cell resistance in a human colorectal carcinoma cell line. *Cancer Res.,* **50:** 2518-2523.

Barrett-Lee, P., Travers, M., Luqmani, Y. and Coombes, R. C., (1990). Transcripts for transforming growth factors in human breast cancer: clinical correlates. *Brit. J. Cancer,* **61:** 612.

Basta, P. V., Sherman, P. A. and Ting, J. P.-Y., (1988). Detailed delineation of an IFN-γ-responsive element important in human HLA-DRα gene expression in a glioblastoma multiform line. *Proc. Nat. Acad. Sci. (USA)* **85:** 8618-8622.

Baxevanis, C., Thanos,D., Reclos, G., Anastasopoulos, E., Tsokos, G., Papamatheakis, J. and Papamichail, M., (1992). Prethymosin enhances human and murine MHC class II surface antigen expression and messenger RNA accumulation. *J. Immunol.,* **148:** 1979-1984.

Benacerraf, B., (1981). Rôle of MHC gene products in immune regulation. *Science.* **212:** 1229.

Benoist, C. and Mathis, D., (1990). Regulation of major histocompatibility complex class II genes: X, Y and other letters of the alphabet. *Ann. Rev. Immunol.,* **8:** 681-715.

Benveniste, E. N., Vidovic, M, Panek, R. B., Norris, J. G., Reddy, A. T. and Benos, D. J., (1991). Interferon-γ-induced astrocyte class II MHC gene expression is associated with both PKC activation and Na^+ entry. *J. Biol. Chem.,* **266:** 18119-18126.

Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan De Vries, M., van Boom, J. H., van der Eb, A. and Vogelstein, B., (1987). Prevalence of *ras* gene mutations in human colorectal cancers. *Nature,* **327:** 293-297.

Bos, J. L., (1989). *ras* oncogenes in human cancer: a review. *Cancer Res.,* **49:** 4682-4689.

Brown, A. M., Barr, C. L. and Ting, J. P.-Y., (1991). Sequences homologous to class II MHC W, X and Y elements mediate constitutive and IFN-γ-induced expression of human class II-associated invariant chain. *J. Immunol.* **146:** 3183-3189.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C., (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature,* **364:** 33-39.

Caldenhoven, E., Coffer, P., Yuan, J., Van de Stolpe, A., Horn, F., Kruijer, W and Van der Saag, P. T., (1994). Stimulation of the human intercellular adhesion molecule-1 promoter by interleukin-6 and interferon-γ involves binding of distinct factors to a palindromic response element. *J. Biol. Chem.* **269:** 21146-21154.

Chen, C. and Okayama, H., (1987), High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:** 2745-2752

Coffey, R. J., Goustin, A. S., Soderquist, A. M., Shipley, G. D., Wolfshol, J., Carpenter, G. and Moses, H. L., (1987). Transforming growth factor α and β expression in human colon cancer lines: implications for an autocrine model. *Cancer Res.*, **47:** 4590-4594.

Cogswell, J. P., Basta, P. V. and Ting, J. P.-Y., (1990). X box-binding proteins positively and negatively regulate transcription of the HLA-DRα gene through interaction with discrete upstream W and V elements. *Proc. Nat. Acad. Sci. (USA)* **87:** 7703-7707.

Cogswell, J. P., Austin, J. and Ting, J. P.-Y., (1991). The W element is a positive regulator of HLA-DRA transcription in various DR^+ cell types. *J. Immunol.* **146:** 1361-1367.

Cogswell, J. P., Zelznik-Le, N. and Ting, J. P.-Y., (1991). Transcriptional regulation of the HLA-DRA gene. *Crit. Rev. Immunol.*, **11:** 87-112.

Collins, T., Korman, A. J., Wake, C. T., Boss, J. M., Kappes, D. J., Fiers, W., Ault, K. A., Gimborne, M. A., Strominger, J. L. and Pober, J. S., (1984). Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. *Proc. Nat. Acad. Sci. (USA),* **81:** 4917-4924.

Connolly, J. M., Hansen, T. H., Ingold, A. L. and Potter, T. A., (1990). Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class I alpha 3 domain: CD8 and the T-cell receptor recognise the same class I molecule. *Proc. Nat. Acad. Sci (USA),* **87:** 2137-2141.

Czarniecki, C. W., Chiu, H., Wong., McCabe, S. and Palladino, M. A., (1988). Transforming growth factor β_1 alters the expression of class II histocompatibility antigens on human cells. *J. Immunol.,* **140:** 4217-4223.

Darley, R., Morris, A. G., Passas, J. and Bateman, W. J., (1993). Interactions between interferon γ and retinoic acid with transforming growth factor β in the induction of immune recognition molecules. *Cancer Immunol. Immunother.* **37:** 112-118.

Darley, R. and Morris, A. G., (1993). Sequential changes in MHC antigen expression induced by the v-Ki-*ras* oncogene. *Cancer Immunol. Imunother.*

Darnell, J. E., Kerr, I. M. and Stark, G. R., (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signalling proteins. *Science,* **264:** 1415-1421.

Das, H. K., Lawrance, S. K. and Weissman, S. M., (1983). Structure and nucleotide sequence of the heavy chain gene of HLA-DR. *Proc. Nat. Acad. Sci. (USA),* **80:** 3543-3547.

Dausset, J., (1981). The major histocompatibility complex in man. *Science,* **213:** 1469.

Decker, T., Lew, D. J., Cheung, Y. -S., Levy, D. E. and Darnell Jr., J. E., (1989). Interaction of α- and γ-interferon in the transcriptional regulation of the gene encoding a guanylate binding protein. *EMBO J.,* **8:** 82009-2014.

Decker, T., Lew, D. J., Mirkowitch, J. and Darnell Jr., J. E., (1991). Cytoplasmic activation of GAF, an interferon-γ regulated DNA binding factor. *EMBO J.,* **10:** 927-932.

Dedrick, R. L. and Jones, P. P., (1990). Sequence elements required for activity of a murine major histocompatibility complex class II promoter bind common and cell-type-specific nuclear factors. *Mol. Cell Biol.,* **10:** 593-604.

Devajyothi, C., Kalvakolanu, I., Babcock, G. J., Vasavada, H. A., Howe, P. H. and Ransohoff, R. M., (1993). Inhibition of the interferon-γ-induced major histocompatibility complex class II gene transcription by interferon-β and type β_1 transforming growth factor in human astrocytes: *definition of a cis-element. J. Biol. Chem.,* **268:** 18794-18800.

Didier, D. K., Schiffenbauer, J., Woulfe, S. L., Zacheis, M. and Schwartz, B. D., (1988). Characterisation of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc. Nat. Acad. Sci. (USA),* **85:** 7322-7326.

Donnellan, I. M., Cantrill, J., Fraser, I. and Morris, A. G., (1995). Activation by IFN-γ of expression of ICAM-1 and MHC class II antigens from colorectal carcinoma. *J. Clin. Pathol.: Mol. Pathol.* **48:** 40-46.

Dressler, G. R., (1989). An up-date on the vertebrate homeobox. *Trends in Genetics,* **5:** 129-130.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A. and Springer, T. A., (1986). Induction by IL-1 and IFN-γ: tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). *J. Immunol.* **137:** 245-254.

Eastman, A., (1987). An improvement to the novel rapid assay for chloramphenicol acetyl transferase gene expression. *BioTechniques* **5:** 730-732

Epstein, S. P., Baer, R., Thornbeck, G. and Belsito, D., (1991). Immunosuppressive effects of transforming growth factor-β: inhibition of the induction of Ia on Langerhans cells by cytokines and of the contact hypersensitivity response. *J. Invest. Dermatol.*, **96:** 832-837.

Faisst, S and Meyer, S., (1992). Compilation of vertebrate-encoded transcription factors. *Nuc. Acids Res.* **20:** 3-26.

Feldmann, M., (1989). Molecular mechanisms involved in human autoimmune diseases: Relevance of chronic antigen presentation, class II expression and cytokine production. *Immunol.*, ***(Suppl 2)***, 66-73.

Figdor, C. G., van Kooyk, Y. and Keizer, G. D., (1990). On the mode of action of LFA-1. *Immunol. Today* **11:** 277-280.

Fogh, J. and Trempe, G., (1975). "Human tumor cells *in vitro* (pp 115-159)." J. Fogh (Ed.), Plenum Press, New York.

Fogh, J., *et al.*, (1977). *J. Nat. Cancer Inst.*, **58:** 209-214.

Forrester, K., Almoghera, C., Han, K., Grizzle, W. E. and Perucho. M., (1987). Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature,* **327:** 298-303.

Fu, X. Y., Kessler, D. S., Veals, S. A., Levy, D. E. and Darnell Jr., J. E., (1990). ISGF-3, the transcriptional activator induced by interferon-α, consists of multiple interacting polypeptide chains. *Proc. Nat. Acad. Sci. (USA),* **87:** 8555-8559.

Goodwin, G. H., (1990). "The analysis of sequence-specific DNA-binding proteins in cell extracts" Gel electrophoresis of nucleic acids: 225-247. Second edition (Eds.: Rickwood, D and Hames, B. D. Series eds.: Rickwood, D. and Hames, B. D., Practical Approach Series). IRL Press, Oxford.

Gorer, P. A., (1937). The genetic and antigenic basis of tumour transplantation. *J. Pathol. Bacteriol.,* **44:** 691

Gorman, C., (1985). High efficiency gene transfer into mammalian cells. DNA cloning volume II. 1st ed. (Ed. Glover, D. M.) (Series Eds: Rickwood, D. and Hames, B. D., Practical Approach Series.) IRL Press, Oxford. 143-190.

Gummina, R. J., Freirè-Moar, J., De Young, L., Webb, D. R. and Devens, B. H., (1991). Transduction of the IFN-γ signal for HLA-DR expression in the promonocytic line THP-1 involves a late-acting PKC activity. *Cellular Immunol.,* **138:** 265-279.

Hai, T., Horikoshi, M., Roeder, R. G. and Green, M. R., (1988). Analysis of the rôle of the transcription factor ATF in the assembly of functional preinititiation complex. *Cell,* **54:** 1043-1051.

Hämmerling, G. J. and Moreno, J., (1990). The function of the invariant chain in antigen presentation by MHC class II molecules. *Immunol. Today* **11:** 337-339.

Hennighausen, L and Lubon, H., (1987). "Interaction of protein with DNA *in vitro*" Guide to molecular cloning techniques: 721-723. First edition: eds: Berger, S. L. and Kimmel, A. R. (Series Eds: Abelson, J. N. and Simon, M. I; *Methods in Enzymology*, **152**). Academic Press, London.

Horikoshi, M., Hai. T., Young-Sun, L., Green M. R. and Roeder, R. G., (1988). Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. *Cell,* **54:** 1033-1042.

Hume, C. R., Accolla, R. S. and Lee, J. S., (1987). Defective HLA class II expression in a regulatory mutant is partially complemented by activated *ras* oncogenes. *Proc. Nat. Acad. Sci. (USA)* **84:** 8603-8607.

Ivashkiv, L. and Glimcher, L. H., (1991). Repression of class II major histocompatibility complex genes by cyclic AMP is mediated conserved promoter elements. *J. Exp. Med.,* **174:** 1583-1592.

Kara, C. J. and Glimcher, L. H., (1991). *In vivo* footprinting of MHC class II genes: bare promoters in the bare lymphocyte syndrome. *Science* **252:** 709-712.

Kara, C. J. and Glimcher, L. H., (1993). Promoter accessibility within the environment of the MHC is affected in class II-deficient combined immunodeficiency. *The EMBO J.* **12:** 187-193.

Kelly, A. and Trowsdale, J., (1985). Complete nucleotide sequence of a functional HLA-DPβ gene and the region between the DPβ1 and DPα2 genes: comparison of the 5' ends of HLA class II genes. *Nuc. Acids Res.,* **13:** 1607-1621.

Kerr, L. A. P., Navsaria, H. A., Barker, J. N. W. N., Sakkas, L. I., Leigh, I. M., MacDonald, D. M. and Walsh, K. I., (1990). Interferon-γ activates coordinate transcription of HLA-DR, DQ and DP genes in cultured keratinocytes and requires *de novo* protein synthesis. *J. Invest. Dermatol.,* **95:** 653-656.

Koerner, T., Hamilton, T and Adams, D., (1987). Suppressed expression of surface Ia on macrophages by lipopolysaccharide: evidence for regulation at the level of accumulation of mRNA. *J. Immunol.,* **139:** 239-243.

La Thangue, N. B. and Rigby, P. W. J., (1988). "*Trans*-acting protein factors and the regulation of eukaryotic transcription". Transcription and splicing (Eds: Hames, B. D. and Glover, D. M.). Frontiers in molecular biology, IRL Press, Oxford.

Landschultz, W. H., Johnson, P. F. and McKnight, S. L., (1988). *Science,* **240:** 1759

Latron, F., Moots, R., Rothbard, J. B., Garrett, T. P. J., Strominger, J. L., and McMichael, A., (1991). Positioning of a peptide in the cleft of HLA-A2 by complementing amino acid changes. *Proc. Nat. Acad. Sci. (USA),* **88:** 11325-11329.

Latron, F., Pazmany, L., Morrison, J., Moots, R., Saper, M. A., McMichael, A. and Strominger, J. L., (1992). A critical rôle for conserved resides in the cleft of HLA-A2 in presentation of a nonapeptide to T cells. *Science,* **257:** 964-967

Lee, Y. J., Panek, R. B., Huston, M. and Benveniste, E. N., (1995). Rôle of protein kinase C and tyrosine kinase activity in IFN-γ-induced expression of the class II MHC. *Am. J. Physiol.,* **268:** 127-137.

Liou, H.-C., Boothby, M. R., Finn, P. W., Davidon, R., Nabavi, N., Zeleznik-Le, N., Ting. J. P.-Y. and Glimcher, L. H., (1990). A new member of the leucine zipper class of proteins that binds to the HLA-DRα promoter. *Science* **247:** 1581-1584.

Ling, P. D., Warren, M. and Vogel, S. L., (1985). Antagonistic effect of interferon-αβ on the interferon-γ induced expression of Ia antigens in murine macrophages. *J. Immunol.,* **135:** 1857-1862.

Look, D. C., Pelletier, M. R. and Holtzman, M. J., (1994). Selective interaction of a subset of interferon-γ response element-binding proteins with the intercellular adhesion molecule-1 (ICAM-1) gene promoter controls the pattern of expression on epithelial cells. *J. Biol. Chem.* **269:** 8952-8958.

Loughlin, A., Woodroofe, M. and Cuzner, M., (1993). Modulation of interferon gamma induced major histocompatibility complex class II and Fc receptor expression on isolated microglia by transforming growth factor beta 1, interleukin 4, noradrenaline and glucocorticoids. *Immunol.,* **79:** 125-130.

Marsh, S. G. E. and Bodmer, J. G., (1992). HLA class II nucleotide sequences, 1991. *Tiss. Antigens,* **37:** 181-189.

Marx, J. L., (1988). Homeobox linked to gene control. *Science,* **242:** 1008-1009.

Matsushima, G. K., Itoh-Lindstrom, Y. and Ting, J. P.-Y., (1992). Activation of HLA-DRA gene in primary human blood T-lymphocytes: novel usage of TATA and the X/Y promoter elements. *Mol. Cell Biol.,* **12:** 5610-5619.

McDonald, G. and Jewell, D., (1987). *J. Clin. Path.,* **40:** 312-317.

Möller, P., Momberg, F., Koretz, K., Moldenhauer, G., Herfarth, C., Otto, H., Hammerling, G. and Schlag, P., (1991). Influence of major histocompatibility complex class I and II antigens on survival in colorectal cancer. *Cancer Res.* **51:** 729-736.

Moots, R. J., Masui, M., Pazmany, L., McMichael, A. J. and Frelinger, J. A., (1991). A cluster of mutations in HLA-A2 α2 helix abolishes peptide recognition by T cells. *Immunogenetics,* **34:** 141-148.

Moots, R. J., Samberg, N. L., Pazmany, L., Frelinger, J. A., McMichael, A. J. and Stauss, H. J., (1992). A cross-species functional interaction between the murine major histocompatibility complex class I alpha 3 domain and human CD8 revealed by peptide-specific cytotoxic T lymphocytes. *Eur. J. Immunol.,* **22:** 1643-1646.

Morris, A. G., and Ward, G. A. (1987). "Production of recombinant interferons by expression in heterologous cells" Lymphokines and interferons: a practical approach, pages 61-71. First edition, eds.: Clemens, M. J., Morris, A. G. and Gearing, A. J. H. (Series eds: Rickwood, D and Hames, B. D. Practical Approach series). IRL press, Oxford.

Morris, A. G. and Tomkins, P. T. (1989). Interactions of interferons in the induction of histocompatibility antigens in mouse fibroblasts and glial cells. *Immunology* **67:** 537-542.

Morris, A. G., Hewitt, C. and Young, S., (1994). The major histocompatibility complex: its genes and their rôles in antigen presentation. *Mol. Aspects Med.* **15:** 377-503.

Morris, A. G. (1995). "Cytokines". Encyclopaedia of Molecular Biology: Fundamentals and Applications (Ed: Meyers, R. A.,). VCH press, New York. *In press.*

Moses, H., Panek., R. B., Benveniste, E. N. and Ting. J. P.-Y., (1992). Usage of primary cells to delineate IFN-γ-responsive DNA elements in the HLA-DRA promoter and to identify a novel IFN-γ-enhanced nuclear factor. *J. Immunol.,* **148:** 3643-3651.

Moss, P. A. H., Moots, R. J., Rosenberg, W. M. C., Rowlandjones, S. J., Bodmer, H. C., McMichael, A. J. and Bell, J. I., (1991). Extensive conservation of alpha-chain and beta-chain of human T cell antigen receptor recognising HLA-A2 and influenza-A matrix peptide. *Proc. Nat. Acad. Sci. (USA),* **88:** 8987-8990.

Müller, M., Briscoe, J., Laxton, C., Guschin, D., Ziemiecki, A., Silvennoinen, O., Harpur, A. G., Barbieri, G., Witthuhn, B. A., Schindler, C., Pellegrini, A., Wilks, A. F., Ihle, J. N., Stark, G. R. and Kerr, I. M., (1993). The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. *Nature,* **366:** 129-135.

Neefjes, J. J. and Momberg, F., (1993). Cell biology of antigen presentation. *Current Opinion Immunol.* **5:** 27-34.

Nelson, C. A., Roof, R. W., McCourt, D. W. and Unanue, E. R., (1992). Identification of the naturally processed form of hen egg-white lysozyme bound to the murine major histocompatibility complex class II molecule I-a(k). *Proc. Nat. Acad. Sci. (USA),* **89:** 7380-7383.

Okada, K., Boss, J. M., Prentice, H., Spies, T., Mengler, R., Auffray, C., Lillie, J., Grossberger, D. and Strominger, J., (1985). Gene organisation of DC and DX subregions of the human major histocompatibility complex. *Proc. Nat. Acad. Sci. (USA),* **82:** 3410

Ono, S. J., Bašil, V., Levi, B.-Z., Ozato, K. and Strominger, J. L., (1991). Transcription of a subset of human class II MHC genes is regulated by a nucleoprotein complex that contains c-fos or an antigenically related protein. *Proc. Nat. Acad. Sci. (USA)* **88:** 4304-4308.

Ono, S. J., Liou, H.-C., Davidon, R., Strominger, J. L. and Glimcher, L. H., (1991). Human X-box-binding protein 1 is required for the transcription of a subset of human class MHC genes and forms a heterodimer with c-fos. *Proc. Nat. Acad. Sci. (USA)* **88:** 4309-4312.

Panek, R. B., Lee, Y. J. and Benveniste, E. N., (1995). TGF-β suppression of IFN-γ-induced class II MHC gene expression does not involve inhibition of phosphorylation of JAK1, JAK2, or signal transducers and activators of transcription, or modification of IFN-γ enhanced factor expression. *J. Immunol.,* **154:** 610-619.

Peterlin, B. M. and Andersson, G., (1990). NF-X2 that binds to the DRA X2 box is AP-1: expression cloning of c-jun. *J. Immunol.,* **145:** 3456-3462.

Piccinini, L., Roman, S. and Davies, T., (1987). autoimmune thyroid disease and thyroid class II major histocompatibility antigens. *Clin. Endo.*, **26:** 253-272.

Ploegh, H. L., Orr, H. T. and Strominger, J. L., (1981). Major histocompatibility antigens: the human (HLA-A, -B, -C) and murine (H2K, H-2D) class I molecules. *Cell.* **24:** 287.

Reimold, A. M., Kara, C. J., Rooney, J. W. and Glimcher, L. H., (1993). Transforming growth factor β_1 repression of the HLA-DRα gene is mediated by conserved proximal promoter elements. *J. Immunol.,* **151:** 4173-4182.

Reith, W., Satola, S., Sanchez, C. H., Amaldi, I., Lisowska-Grospiere, B., Griscelli, C., Hadam, M. R. and Mach, B., (1988). Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. *Cell,* **53:** 897-906.

Roitt, I., (1994). Essential Immunology, 8th edition. Blackwell Scientific Publications.

Rosa, F., Hafat, D., Abadie, A., Wallach, D., Revel, M. and Felous, M., (1983). Differential regulation of HLA-DR mRNAs and cell surface antigens by interferon. *EMBO J.,* **2:** 1585-1589.

Rotzchke, O. and Falk, K. (1991). Naturally occurring peptide antigens derived from the MHC class I-restricted processing pathway. *Immunol. Today,* **12:** 447-455.

Rudensky, A. Y., Prestonhurlburt, P., Hong, S. C., Barlow, A. and Janeaway, C. A., (1991). Sequence analysis of peptides bound to MHC class II molecules. *Nature,* **353:** 622-627.

Sakurai, M. and Strominger, J. L., (1988). B-cell-specific enhancer activity of conserved upstream elements of the class II MHC DQB gene. *Proc. Nat. Acad, Sci. (USA),* **84:** 8598.

Salter, R. D., Benjamin, R. J., Wesley, P. K., Buxton, S. E., Garrett, T. P., Clayberger, C., Krensky, A. M., Norment, A. M., Littman, D. R., and Parham, P., (1990). A binding site for the T cell coreceptor CD8 on the alpha 3 domain of HLA-A2. *Nature,* **345:** 41-46.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (Eds) (1989). Molecular cloning: a laboratory manual. Second edition **vols. I-III.** Cold Spring Harbor laboratory press, New York.

Semple, T. U., *et al.,* (1978). *Cancer Res.,* **38:** 1345-1355.

Sherman, P. A., Basta, P. V. and Ting, J. P.-Y., (1987). Upstream DNA sequences required for tissue-specific expression of the HLA-DRα gene. *Proc. Nat. Acad. Sci. (USA)* **84:** 4254-4258.

Sherman, P. A, Basta, P. V., Heguy, A., Wloch, M. K., Roeder, R. G. and Ting, J. P.-Y., (1989). The octamer motif is a B-lymphocyte-specific regulatory element of the HLA-DRA gene promoter. *Proc. Nat. Acad. Sci. (USA),* **86:** 6739-6743.

Sherman, P. A., Basta, P. V., Moore, T. L., Brown, A. M. and Ting, J. P.-Y., (1989). Class II box consensus sequences in the HLA-DRα gene: transcriptional function and interaction with nuclear proteins. *Mol. Cell Biol.* **9:** 50-56.

Sloan, J H., Hasegawa, S. L. and Boss, J. M., (1992). Single base pair substitutions within the HLA-DRA promoter separate the functions of the X1 and X2 boxes. *J. Immunol.,* **148:** 2591-2599.

Snell, G. D., (1981). Studies in histocompatibility. *Science,* **213:** 172.

Snyder, D., Beller, D. and Unanue, E., (1982). Prostaglandins modulate macrophage Ia expression. *Nature,* **299:** 163-165.

Spier, C. M., Grogan, T. M., Lippman, S. M., Slymen, D. J., Rybski, J. A. and Miller, T. P., (1988). The aberrancy of immunophenotype and immunoglobulin status as indicators of prognosis in B cell diffuse large cell lymphoma. *Am. J. Pathol.,* **133: 118-126.**

Stade, B. G., Messer, G., Riethmüller, G. and Johnson, J. P., (1990). Structural characteristics of the 5' region of the human ICAM-1 gene. *Immunobiol.* **182:** 78-87.

Steimle, V., Siegrist, C.-A., Mottet, A., Lisowska-Grospierre, B. and Mach, B., (1994). Regulation of MHC class II expression by interferon-γ mediated by the transactivator gene CIITA. *Science,* **265:** 106-109.

Swingler, S. M., (1992). Cytokine regulation of HIV type 1 gene expression in cells of neural origin. PhD thesis, University of Warwick.

Struhl, K., (1989). Helix-turn-helix, zinc finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins. *Trends in Biochemical Sciences,* **14:** 137-140.

Sullivan, K. E., Calman, A. F., Nakanishi, M., Tsang, S. Y., Wang, Y. and Peterlin, B. M., (1987). A model for the transcriptional regulation of MHC class II genes. *Immunol. Today* **8:** 289-293.

Terhorst, C., Alarcon, B., de Vries, J., and Spits, H., (1988). "T-lymphocyte recognition and activation". *Molecular Immunology* (Eds: Hames, B. D. and Glover, D. M.). Frontiers in molecular biology, IRL Press, Oxford.

Ting, J. P.-Y. and Baldwin, A. S., (1993). Regulation of MHC gene expression. *Current Opinion Immunol.* **5:** 8-16.

Ting, J. P.-Y., (1993). Current concepts in DRA gene regulation. *Immunol. Res.,* **12:** 65-77.

Todd, I., Hammond, L., James, R., Feldmann, M. and Bottazzo, G., (1990). Epidermal growth factor and transforming growth factor alpha suppress HLA class II induction in human thyroid epithelial cells. *Immunology,* **69:** 91-96.

Todd, I., Pujol-Borrell, R., Hammond, L., McNally, J., Feldmann, M. and Bottazzo, G., (1987). Enhancement of thyrocyte HLA class II expression by thyroid stimulating hormone. *Clin. Exp. Immunol.,* **69:** 524-529.

Tom, B. H., *et al.,* (1976). *In vitro,* **12:** 180-191.

Traugott, U., (1987). Multiple sclerosis: Relevance of class I and class II MHC expressing cells to lesion development. *J. Neuroimmunol.,* **16:** 283-302.

Tsang, S. Y., Nakanishi, M, and Peterlin, B. M., (1988). B-cell-specific and interferon-γ-inducible regulation of the HLA-DRA gene. *Proc. Nat. Acad. Sci. (USA),* **85:** 8598-8602.

Tsang, S. Y., Nakanishi, M. and Peterlin, B. M., (1990). Mutational analysis of the DRA promoter: *cis*-acting sequences and *trans*-acting factors. *Mol. Cell Biol.* **10:** 711-719.

Vignali, D. A. A., Urban, R. G., Chicz, R. M. and Strominger, J. L., (1993). Minute quantities of a single immunodominant foreign epitope are presented as large nested sets by major histocompatibility complex class II molecules. *Eur. J. Immunol.,* **23:** 1602-1607.

Vilen, B. J., Cogswell, J. P. and Ting, J. P.-Y., (1991). Stereospecific alignment of the X and Y elements is required for MHC class II DRα promoter function. *Mol. Cell Biol.* **11:** 2406-2415.

Vilen, B. J., Penta, J. F., and Ting, J. P-Y., (1992). Structural constraints within a trimeric transcriptional regulatory region: constitutive and interferon-γ inducible expression of the HLA-DRA gene. *J. Biol. Chem.,* **267:** 23728-23734.

Wright, K. L., and Ting, J. P.-Y., (1992). *In vivo* footprinting analysis of the HLA-DRA gene promoter: cell-specific interaction at the octamer site and up-regulation of X box binding by interferon γ. *Proc. Nat. Acad. Sci. (USA),* **89:** 7601-7605.

Wright, K. L., Vilen, B. J., Itoh-Lindstrom, Y., Moore, T. L., Li, G., Criscitello, M., Cogswell, P., Bradley Clarke, J. and Ting, J. P.-Y., (1994). CCAAT box binding protein NF-Y facilitates *in vivo* recruitment of upstream DNA binding transcription factors. *EMBO J.,* **13:** 4042-4053.

Yang, Z., Sugawara, M., Ponath, P. D., Wessendorf, L., Banerij, J., Li, Y. and Strominger, J. L., (1990). Interferon-gamma response region in the promoter of the human DPA gene. *Proc. Nat. Acad. Sci. (USA),* **87:** 9226-9230.

Zaloudik, J., Moore, M., Ghosh, A. K., Mechl, Z. and Rejthar, A. (1988). DNA content and MHC class II antigen expression in malignant melanoma: clinical course. *J. Clin. Pathol.*, **41:** 1078-1084.

Zhou, H. and Glimcher, L. H., (1995). Human MHC class II gene transcription directed by the carboxyl terminus of CIITA, one of the defective genes in type II MHC combined immune deficiency. *Immunity*, **2:** 545-553.

APPENDIX: list of suppliers

APPENDIX A: LIST OF SUPPLIERS

Amersham International plc., Aylesbury, Buckinghamshire, UK.

^{32}P radioisotopes, restriction enzymes

BDH Laboratory Supplies, Ltd., Fourways, Carlyon Industrial Estate, Atherstone, Warwickshire, UK.

General chemicals

Bio-Rad Laboratories, Ltd., Caxton Way, Watford Business Park, Watford, UK.

Protein assay reagent

Boehringer Mannheim, Ltd., Bell Lane, Lewes, East Sussex, UK.

Calf intestine alkaline phosphatase, *E. coli* tRNA, restriction enzymes

British Biotechnology, Ltd., Barton Lane, Abingdon, Oxon., UK.

Human transforming growth factor β (TGF-β)

Camlab, Ltd., Nuffield Road, Cambridge, UK.

Phenol, phenol/chloroform

Costar, UK, Ltd., Victoria House, Desborough Street, High Wycombe, Bucks, UK.

Cell-freezing vials, tissue culture flasks, cell scrapers

Difco Laboratories, Ltd.,

Bacto-agar, bacto-tryptone

Du-Pont (UK), Ltd., NEN Research Products, Wedgewood Way, Stevenage, Herts, UK.

[^{3}H]-acetyl Coenzyme A, Econofluor scintillation fluid

Eastman Kodak, Ltd., Acornfield Road, Knowsley Industrial Park North, Liverpool, UK.

135mm transparency and print film, X-ray developer and fixative, photgraphic paper, N'N-methylene *bis*-acrylamide

Fisons, Ltd., Bishop Meadow Road, Loughborough, Leicestershire, UK.

Acrylamide, butan-1-ol, chloroform, dimethyl sulphoxide, isoamyl alcohol

Fluka AG, CH-9470, Buchs.

Hydrazine

Fuji Photo Film Company (UK), Ltd., 125, Finchley Road, London, UK.

X-ray film

Gibco-BRL, Trident House, Renfrew Road, Paisley, UK.

DNase I, 1kb DNA ladder, foetal calf serum, trypsin-EDTA, glutamine, penicillin/streptomycin, modification and restriction enzymes

Life Sciences, Inc., 72nd Street North, St. Petersberg, Florida, USA,

AMV reverse transcriptase

May and Baker, Ltd., Barton Road, Eccles, Manchster, UK.

Acetic acid, formic acid

Pharmacia Biosystems, Ltd., Davy Avenue, Knowlhill, Milton Keynes, UK.

Poly (dI:dC)-(dI:dC), restriction enzymes

Polaroid (UK) Ltd., Ashley Road, St. Albans, Herts, UK.

Type 55 land film

Promega (UK) Ltd., Epsilon House, Enterprise Road, Chilworth Research Centre, Southampton, UK.

Modification enzymes, RNase-free DNase I, plasmid pCATbasic

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, UK.

Agarose, ampicillin, CAT enzyme, chloramphenicol, DMEM, ethidium bromide, foetal calf serum, hepes buffer, human IFN-αβ, protease inhibitors: antipain, leupeptin, pepstatin A and PMSF, RPMI 1640 medium

Stratagene, Ltd., Cambridge Innovation Centre, Cambridge Science Park, Milton Road, Cambridge, UK.

Plasmids pBSII-KS$^+$, pSV2CAT